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13. ABSTRACT ***(Maximum 200 Words)***

This grant application was focused on understanding the PTEN tumor suppressor gene, both at a functional level and with respect to the extent of PTEN loss in prostate tumors. The aims of this new investigator award were to develop antibody reagents to the protein product (PTEN), to characterize the function of PTEN through the identification of downstream targets, and to determine, using immunohistochemistry, the extent to which the PTEN protein was lost in primary tumors.

It is now recognized that PTEN is a lipid phosphatase that can antagonize signaling though the phosphoinositide-3 kinase pathway. Our work, funded by the New Investigator mechanism, showed that PTEN can regulate cell-cycle progression by controlling the passage of cells through the G1 checkpoint. This function of PTEN requires its lipid phosphatase activity and requires that PTEN act upstream of the serine-threonine kinase Akt. This kinase, in the absence of PTEN is constitutively active and recent data in our lab further suggests that cell-cycle regulation is linked to the ability of PTEN to inhibit Akt thereby restoring the function of forkhead transcription factors such as FKHR and FKHRL1. Based on this data, in collaboration with a local biopharmaceutical company, we are working on developing inhibitors of the Akt kinase family. Here, our hypothesis is that PTEN null cells may be exquisitely dependent upon Akt for either proliferation or survival.

In collaboration with Dr. Massimo Loda, we studied the expression of the PTEN protein in 127 primary prostate tumors obtained by radical prostatectomy. In these studies we found that loss of PTEN was highly associated with higher Gleason score and with more advanced stage tumors. In total these data highlight the importance of PTEN loss to the development and progression of prostate cancer, and the hope that Akt inhibitors will be particularly beneficial in this group of tumors.

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TABLE OF CONTENTS

Introduction

The *PTEN* tumor suppressor gene was cloned in 1997 from the 10q23 chromosomal interval (Li and Sun, 1997; Li et al., 1997; Steck et al., 1997). This region of chromosome 10 is frequently altered in prostate cancer and in particular undergoes allelic loss in more advanced and higher grade tumors (Gray et al., 1995). In this grant application, we sought to try and determine the function of the protein product of *PTEN*, to raise antibodies against the protein, and to use such antibodies both in functional studies and to look at the extent to which the PTEN protein is lost in prostate tumors. We have been successful in each of these goals as will be described below.

Body

Statement of Work (original)

Task 1. Generation of Specific Antibodies

a) Develop and affinity purify the rabbit anti-sera

b) Obtain clonal hybridoma lines producing PTEN specific antibodies

c) Optimize conditions for these various reagents in IBs, IPs, immunofluorescence and IHC techniques using *PTEN* positive and negative cell lines.

Completed (see Ramaswamy et. al. PNAS. 1999, 96:2110-2115)

Task 2: Characterize the endogenous protein

a and b) sub cellular localization as determined by immunofluorescence and fractionation

c) post-translational modifications as determined by IP/western blots, IP/S^{35} labeling and ^{32}P labeling

d) Protein regulation as determined by steady-state protein levels in cells at various points in the cell-cycle and as a functions of adhesion or non-adhesion, and as a function of adhesion to various substrates.

Completed (unpublished, and Vazquez et. al. Mol Cell Biol. 2000, 20:5010-8)

Task 3:.Characterize putative substrates

a) Collect panel of PTEN+/+ and PTEN-/- cell lines

b) Generate substrate trapping, tumor-derived and deletion mutants of PTEN in mammalian expression vectors.

c) Generate the same mutants in as chimeras of glutathione-S-transferase

d) Produce wild-type and mutant variants on PTEN in cells and in bacteria

e) Test GST-PTEN for phosphatase activity in vitro

f) Generate cell lines which stably express or express an inducible form of PTEN

g) Attempt to identify candidate substrates using GST-PTEN and substrate trapping derivatives thereof as affinity reagents.

h) Attempt to identify candidate substrates using over expressed HA-tagged PTEN and mutant derivatives thereof as affinity reagents.

i) Attempt to identify interacting proteins bound to the endogenous PTEN.

j) Attempt to clone putative targets

Completed (see Ramaswamy et. al. PNAS. 1999, 96:2110-2115 and Nakamura et. al., Mol Cell Biol, in press)

Task 4: Screen prostate tumors for PTEN protein by immunohistochemistry(IHC) with multiple antibodies.

a and b) Develop murine and rabbit polyclonal reagent for IHC

c) Develop monoclonal antibodies for IHC **(Incomplete)**

d) Test antibody reagents against PTEN +/+ and PTEN-/- cells using paraffin embedded cells.

e) Test antibody reagents against tumor samples with an intact versus a deleted PTEN allele.

f) Perform IHC tests on tissue samples from tumor banks. Correlate with stage, grade and organ confinement.

Completed (see McMenamin et. al. Cancer Res. 1999, 59:4291-6).

Summary of Work Accomplished (Listed by Aim)

Aim 1: The identification, and characterization of the protein product of the *PTEN* tumor suppressor gene

Rabbits and mice were immunized with GST-PTEN fusion proteins produced in *e.coli*. Fusion proteins encoding the PTEN C-terminus, the PTEN N-terminus and the full-length protein were used. Anti-sera from rabbits that have been generated include D7 and D8 to the amino terminus, C54 and C55 to the C-terminus and C-56 to the full-length protein. In addition, several monoclonal antibodies have been generated including PC-15, PC-17 and PC-22.

Of these antisera and antibodies C54 is the highest titer antibody. This antibody specifcially recognizes a 58 Kd protein on immunoblots that co-migrates with IVT PTEN (data not shown). Furthermore, immunoprecipitation with C54, PC15, D7 or C56 followed by western blotting with C54 recognizes the same protein (Ramaswamy et al., 1999) and data not shown. Further, this protein species is absent from cells that are known to have sustained truncating mutations or biallelic deletion of *PTEN*. Thus, this protein is the endogenous PTEN protein. C54 and PC15 antibody reagents have been licensed and are commercially available through a number of vendors.

In the course of our work it became clear that recombinant PTEN can act as a lipid and protein phosphatase(Maehama and Dixon, 1998; Myers et al., 1997). With these antibody reagents we have demonstrated that this is the case of the endogenous protein as well. These antibody reagents are now being used to ask whether PTEN localization is regulated and whether the phosphatase activity of the protein is regulated. Here we have found that PTEN is a phosphoprotein and that site specific phosphorylation of PTEN regulates its activity in a number of assays including the ability of PTEN to induce a cell-cycle arrest.

This work is detailed in our recently published article in Molecular and Cell Biology and is enclosed as a reprint **(Vazquez et. al. Mol Cell Biol. 2000, 20:5010-8)**.

Aim 2: To identify candidate downstream phosphorylated targets of PTEN

PTEN is a protein and lipid phosphatase

The protein product of the *PTEN* gene(PTEN) is a dual-specificity phosphatase. Recombinant PTEN is capable of dephosphorylating both tyrosine and threonine phosphorylated substrates(Myers et al., 1997), and in addition can dephosphorylate phosphatidylinositol 3,4,5-trisphosphate ($PI3,4,5P_3$)(Maehama and Dixon, 1998; Myers et al., 1997), a product of phosphatidylinositol-3-kinase activity (PI3K).

PTEN induces a block in the G1 phase of the cell-cycle

786-0 renal carcinoma cells, which lack PTEN protein, were transiently co-transfected with a plasmid encoding the cell surface marker CD19, and plasmids encoding PTEN or mutant derivatives. The cell-cycle distribution of the CD19+ cells (marking the transfected cells) was determined by staining with FITC-conjugated anti-CD19 and propidium iodide followed by FACS analysis. We found that wild-type PTEN was capable of inducing a G1 block but tumor derived mutants could not(Ramaswamy et al., 1999). We next compared wild-type PTEN to a number of naturally occurring PTEN mutants in the cell-cycle assay, in protein phosphatase assays (using a phospho-tyrosine substrate) and in assays testing dephosphorylation of H^3-1,3,4,5 inositol tetrakisphosphate(H^3-IP_4). This latter assay reflects the ability of PTEN to dephosphorylate $PI3,4,5P_3$ in vivo (Maehama and Dixon, 1998). One particularly informative mutant was found, PTEN;G129E. This mutant was identified in the germline *PTEN* gene of two independent Cowdens families (Marsh et al., 1998). In our assays it retained phosphatase activity against a protein substrate, but was incapable of arresting cells in G1 and was incapable of dephosphorylating lipid substrates as measured by dephosphorylation of H^3-IP_4. Thus, the ability of PTEN to induce a cell-cycle block correlated best with its ability to dephosphorylate a lipid substrate. These data raised the possibility that the regulation of downstream targets of PI3K might be critical for PTEN mediated cell-cycle control. One such downstream effector is the proto-oncogene Akt (Downward, 1998). We first sought to determine whether PTEN could negatively regulate Akt kinase activity. A plasmid encoding T7-epitope tagged Akt was transfected into U2-OS cells with either empty vector or with a plasmid encoding PTEN. PTEN co-transfection led to a dramatic down-regulation of Akt kinase activity as measured by immunoprecipitation of Akt followed by an in vitro kinase assay with an Akt substrate. We next asked whether Akt might be able to override a PTEN induced G1 block. While wild-type Akt had a minimal effect on the PTEN induced G1 block, a myristoylated form of Akt, which no longer requires PI-3,4,5-P_3 for activation, was dramatically better at overcoming a PTEN block. In contrast, kinase-inactive versions of both Akt and Myr-Akt were unable to override PTEN effects. These data suggest that PTEN mediated cell-cycle inhibition depends upon negative regulation of the PI3K/Akt signaling pathway. In keeping with the notion that Akt is critical downstream target of PI3K, tumors that lack PTEN have deregulated Akt activity.

This work is detailed in the enclosed reprint **(Ramaswamy S, et. al., Proc Natl Acad Sci U S A. *96:* 2110-2115, 1999.)**

These data link Akt and PTEN in a cell cycle pathway downstream of PI3K. In support of the idea that the PTEN/PI3K/Akt pathway can regulate the cell cycle, the expression of activated Akt or of activated PI3K in a serum-starved cell is sufficient to induce S-phase entry (Klippel et al., 1998). Furthermore, in *PTEN* heterozygous mice there is an increase in proliferating cells in the prostate and thyroid glands (Di Cristofano et al., 1998; Podsypanina et al., 1999). In addition, in early PTEN-/- embryos there is widespread, excess cellular

proliferation preceding embryonic death (Stambolic et al., 1998). Thus, PTEN is necessary *in vivo* role for the appropriate regulation cell-cycle progression and cellular proliferation.

We have now gone on to show that this cell-cycle arrest function of PTEN appears to be mediated through a group of Forkhead transcription factors (AFX, FKHR and FKHRL1). These factors are regulated by Akt, and in PTEN null cells are constitutively phosphorylated and are thus inactivated. We found that restoration of Forkhead activity was sufficient to induce a G1 arrest in PTEN null through the induction of p27.

This work is detail in the enclosed manuscript, now in press in MCB. **(Nakamura et. al. Mol Cell Biol 2000, in press)**.

Aim 3. To determine whether PTEN immunostaining is of prognostic value in patients with early stage prostate cancer.

A murine polyclonal antiserum was raised against a glutathione-S-transferase fusion polypeptide of the carboxyl terminus of PTEN. Archival paraffin tissue sections from 109 cases of resected prostate cancer were immunostained with the antiserum, utilizing DU145 and PC-3 cells as positive and negative controls respectively. PTEN expression was seen in the secretory cells. Cases were considered positive when granular cytoplasmic staining was seen in all tumor cells; mixed, when areas of both positive and negative tumor cell clones were seen; and negative when no tumor but adjacent benign prostate tissue showed positive staining. Seventeen cases (15.6%) of prostate cancer were positive, 70 (64.2%) were mixed and 22 (20.2%) were negative. Total absence of PTEN expression correlated with Gleason score ($p=0.0081$), correlated more significantly with a Gleason score of 7 or higher ($p=0.0004$) and with advanced pathological stage (American Joint Committee on Cancer (AJCC) T3c, T4) ($p=0.0078$). Thus, loss of PTEN protein is correlated with pathological markers of poor prognosis in prostate cancer (McMenamin et al., 1999)

This work is detail in the enclosed reprint published in 1999 in Cancer Research.**(see McMenamin et. al. Cancer Res. 1999, 59:4291-6)**.

Key Research Accomplishments

1. Developed antibodies to PTEN and characterized the endogenous protein.
2. Discovered that PTEN can regulate cell-cycle progression.
3. Discovered that PTEN is regulated by phosphorylation.
4. Discovered that Forkhead factors are key effectors of PTEN function.
5. Discovered that PTEN protein is lost in 20% of primary prostate tumors, and that such loss is highly correlated with grade and stage.

Reportable Outcomes:

Licenses:

1. Licensed two PTEN antibodies.

Manuscripts:

1. Ramaswamy, S., Nakamura, N., Vazquez, F., Batt, D. B., Perera, S., Roberts, T. M., and **Sellers, W. R.** Regulation of G1 progression by the PTEN tumor suppressor protein is linked to inhibition of the phosphatidylinositol 3-kinase/Akt pathway, Proc Natl Acad Sci U S A. *96:* 2110-2115, 1999.
2. McMenamin, M. E., Soung, P., Perera, S., Kaplan, I., Loda, M., and **Sellers, W. R.** Loss of PTEN expression in paraffin-embedded primary prostate cancer correlates with high Gleason score and advanced stage, Cancer Res. *59:* 4291-6, 1999.
3. Vazquez, F. and **Sellers, W. R**. The PTEN tumor suppressor protein: an antagonist of phosphoinositide 3- kinase signaling, Biochim Biophys Acta. *1470:* M21-M35, 2000.
4. Ramaswamy S, and **Sellers W. R**. *PTEN* a prostate cancer tumor suppressor gene. The Prostate Journal (in press) 2000.
5. Vazquez F. Ramaswams S, Nakamura N and **Sellers, W.R.** Phosphorylation of the PTEN tail regulates stability and the biological function**.** Mol Cell Biol *20:* 5010-5018 2000
6. Nakamura, N., Ramaswamy, S., Vazquez, F., and Sellers, W. R. FKHR is a critical effector of cell death and cell cycle arrest downstream of PTEN, . Mol Cell Biol *(in press):*, 2000.

Clinical Translational research: Signed a sponsored research agreement with Kinetix Pharmaceuticals to develop and test Akt inhibitors.

Grant funding obtained:

CapCURE Award 1998 Developing Akt transgenic mouse models
CaPCURE Award 1999 Purification of a PTEN kinase
ACS RPG grant 2000 The functional analysis of the PTEN tumor suppressor protein (overlap with RO1)
NCI-RO1CA85 2000 The functional analysis of the PTEN tumor suppressor protein

Conclusions

In conclusion, our lab has identified PTEN has an important regulator of proliferation and cell-cycle progression in prostate cells. Furthermore, this PTEN function appears to be linked to its ability to regulate the serine-threonine kinase Akt and the downstream transcription factor FKHR. Based upon this work we are collaborating with a pharmaceutical company to develop Akt kinase inhibitors. I believe such inhibitors will have a high degree of activity in PTEN null prostate tumors. Our studies of primary prostate tumors suggest that loss of PTEN protein by IHC staining is associated with a more aggressive form of prostate cancer. In keeping with these data others have shown that PTEN loss in metastatic foci of prostate cancer approaches 50-60%. If this is true then Akt inhibitors will potentially be useful in exactly the type of tumors that appear destined to relapse following surgery. In addition, these data raise the possibility that PTEN loss may be useful as a prognostic marker.

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Appendices

1. Ramaswamy et. al. PNAS.
2. McMenamin et. al. Cancer Research.
3. Vazquez et. al. MCB.
4. Nakamura et. al. MCB (manuscript enclosed).

Final Report

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McMenamin, M. E., Soung, P., Perera, S., Kaplan, I., Loda, M., and Sellers, W. R. (1999). Loss of PTEN expression in paraffin-embedded primary prostate cancer correlates with high Gleason score and advanced stage. Cancer Res *59*, 4291-6.

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Personnel:

Personnel receiving salary from this grant during the past two years included, Dr. William R. Sellers, the principal investigator, Dr. Shivapriya Ramaswamy PhD, and Ms. Sauni Perera.

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Cell Biology

Regulation of G_1 progression by the *PTEN* tumor suppressor protein is linked to inhibition of the phosphatidylinositol 3-kinase/Akt pathway

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Communicated by Kurt J. Isselbacher, Massachusetts General Hospital, Charlestown, MA, December 29, 1998 (received for review October 30, 1998)

ABSTRACT ***PTEN/MMAC1* is a tumor suppressor gene located on chromosome 10q23. Inherited *PTEN/MMAC1* mutations are associated with a cancer predisposition syndrome known as Cowden's disease. Somatic mutation of PTEN has been found in a number of malignancies, including glioblastoma, melanoma, and carcinoma of the prostate and endometrium. The protein product (PTEN) encodes a dual-specificity protein phosphatase and in addition can dephosphorylate certain lipid substrates. Herein, we show that PTEN protein induces a G_1 block when reconstituted in *PTEN*-null cells. A *PTEN* mutant associated with Cowden's disease (PTEN;G129E) has protein phosphatase activity yet is defective in dephosphorylating inositol 1,3,4,5-tetrakisphosphate *in vitro* and fails to arrest cells in G_1. These data suggest a link between induction of a cell-cycle block by PTEN and its ability to dephosphorylate, *in vivo,* phosphatidylinositol 3,4,5-trisphosphate. In keeping with this notion, PTEN can inhibit the phosphatidylinositol 3,4,5-trisphosphate-dependent Akt kinase, a downstream target of phosphatidylinositol 3-kinase, and constitutively active, but not wild-type, Akt overrides a PTEN G_1 arrest. Finally, tumor cells lacking PTEN contain high levels of activated Akt, suggesting that PTEN is necessary for the appropriate regulation of the phosphatidylinositol 3-kinase/Akt pathway.**

Abnormalities of chromosomal region 10q23 are frequent in a number of malignancies, including prostate cancer and glioblastoma (1, 2). Recently, a candidate tumor suppressor gene *PTEN/MMAC1/TEP1* (for simplicity hereafter referred to as *PTEN*) was cloned and mapped to this region (3–5). Somatic mutations of *PTEN* are found in a number of malignancies, including glioblastoma, melanoma, and carcinomas of the prostate, lung, endometrium, and head and neck (3, 4, 6–14). Germ-line mutations of the *PTEN* gene are associated with the development of Cowden's disease (CD) and Bannayan–Zonana syndrome (BZS) (15–18). CD is characterized by the occurrence of multiple hamartomas in the skin, gastrointestinal tract, breast, thyroid, and central nervous system and an increased incidence of breast and thyroid cancers (18). BZS is a related syndrome in which intestinal hamartomas are accompanied by neurological abnormalities including mild mental retardation, delayed motor development, vascular malformations, and speckled penis (18).

The predicted protein product of the *PTEN* gene (referred to hereafter as PTEN) has homology to tensin, an actin binding protein localized to focal adhesion complexes (19); to auxilin, a protein involved in the uncoating of clatherin-coated vesicles (20); and to dual-specificity phosphatases (4, 21). Recombinant PTEN is capable of dephosphorylating both tyrosine- and threonine-phosphorylated substrates and in addition can dephosphorylate phosphatidylinositol 3,4,5-trisphosphate (PtdIns-3,4,5-P_3) (22, 23). Overproduction of PTEN can suppress colony formation in certain cells, growth in soft agar, and tumor formation in nude mice (24, 25). Recent data suggest that PTEN might function, at least in part, through regulation of focal adhesion kinase and the subsequent inhibition of adhesion and migration (26). PTEN is essential for murine embryonic development beyond day 7.5. In the mouse loss of *PTEN* allele leads to hyperplasia and dysplasia in the skin, gastrointestinal tract, and prostate, as well as tumor formation (27).

In this study, we found that reintroduction of a PTEN cDNA into cells lacking a wild-type PTEN protein led to a cell-cycle block in G_1. This function was tightly linked to the phosphatase activity of PTEN and was inactivated by tumor-derived mutations. Furthermore, a PTEN mutant, associated with CD, that retains protein phosphatase activity was defective in arresting cells in G_1 and was also defective in dephosphorylating inositol 1,3,4,5-tetrakisphosphate (IP_4).

These data suggested that PTEN might regulate cell-cycle progression by blocking activation of downstream targets of phosphatidylinositol 3-kinase such as the protooncogene Akt. In keeping with this notion, PTEN was capable of inhibiting wild-type Akt kinase activity in cells. Furthermore, a constitutively active form of Akt, but not wild-type Akt, overrode a PTEN-induced cell-cycle block.

MATERIALS AND METHODS

Cell Culture, Transfection, and Metabolic Labeling. ACHN, 786-O, SAOS-2, and U2-OS cells (gifts from the Kaelin laboratory) were maintained in DMEM containing 10% Fetal Clone (HyClone), penicillin and streptomycin at 37°C. Cells were transfected with Fugene 6 (Boehringer-Mannheim) for 786-O cells or by the calcium phosphate procedure for U2-OS, ACHN, and SAOS-2 cells, as described (28, 29). Transfected 786-O cells were metabolically labeled for 3 h in 5 ml of methionine-free medium supplemented with 10% dialyzed fetal calf serum and [^{35}S]methionine (100 μCi/ml; 1 Ci = 37 GBq).

Plasmids. A cDNA fragment encoding PTEN amino acid residues 1–403 was PCR-amplified from a 293 cDNA library (30) and ligated to vector pSG5L-HA (28) to give pSG5L-HA-PTEN;WT. An Akt-1 cDNA was amplified by reverse transcription-coupled PCR from total HeLa cell RNA and reamplified with a 5′ primer containing a Kozak sequence and

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Abbreviations: IP_4, inositol 1,3,4,5-tetrakisphosphate; PtdIns, phosphatidylinositol 3,4,5-trisphosphate; CD, Cowden's disease; HA, hemagglutinin; GST, glutathione *S*-transferase.

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sequences encoding a hemagglutinin (HA) epitope and cloned into pLNCX to give pLNCX-HA-Akt. A double-stranded oligonucleotide encoding the src myristoylation sequence was inserted 5′ of the HA tag to generate pLNCX-Myr-HA-Akt. pSG5L-HA-PTEN;C124S, pSG5L-HA-PTEN;G129R, pSG5L-HA-PTEN;G129E, pSG5L-HA-PTEN;1–274, pSG5L-HA-PTEN;1–336 and pSG5L-HA-PTEN;Δ274–342, pLNCX-HA-Akt;K179M, pLNCX-myr-HA-Akt;K179M were generated by site-directed mutagenesis or by PCR mutagenesis. Inserts from the pSG5L-HA-PTEN plasmids were cloned into pGEX2T to give the corresponding pGEX2T-PTEN plasmids. A cDNA for AKT-1 was PCR-amplified from a fetal brain library and ligated to the vector from pCDNA3-T7-VHL to give pCDNA3-T7-AKT. All plasmid inserts obtained by PCR or altered by site-directed mutagenesis were verified by sequencing. pCD19 has been previously described (31).

Antibodies. Production of PTEN antiserum (C54) will be described elsewhere (32). HA.11, fluorescein isothiocyanate-conjugated anti-CD19, anti-T7, and anti-phospho-Akt (Ser-473) antibodies were obtained from Babco (Richmond, CA), Novagen, Caltag (South San Francisco, CA), and New England Biolabs, respectively.

Immunoprecipitations and Immunoblotting. Preparation of whole cell extracts, immunoprecipitations, and immunoblotting conditions are as described (28). For immunoblotting, C54 antiserum was diluted 1:500 in TBS/4% milk. Secondary antibodies, alkaline phosphatase-conjugated goat anti-mouse or goat anti-rabbit (Southern Biotechnology Associates) were diluted 1:5,000. For chemiluminescent detection, horseradish peroxidase-conjugated anti-mouse antibody (Santa Cruz Biotechnology) was used at a 1:2,000 dilution and detected with the SuperSignal kit (Pierce).

Fluorescence-Activated Cell Sorting. Cells grown on p100 plates were transfected with 4 μg of pCD19 and either 11 μg (Fugene 6 transfections) or 21 μg (calcium phosphate transfections) of the backbone pSG5L plasmid or pSG5L plasmids encoding PTEN or the indicated PTEN mutants. Cell-cycle determination of the CD19+ cells was carried out as described (28).

Protein and Inositol Phosphatase Assays. Poly(Glu_4-Tyr_1) copolymer (Sigma) was phosphorylated *in vitro* essentially as described (22). Briefly, poly(Glu_4-Tyr_1) was resuspended at a final concentration of 3.3 mg/ml in 50 mM Tris·HCl (pH 7.4), 2 mM $MnCl_2$, 10 mM $MgCl_2$, and 0.1 mM ATP in a reaction mixture containing 10 μCi of [γ-^{33}P]ATP and 100 units of β-insulin receptor kinase (Stratagene) and incubated at 30°C for 4 h. Labeled copolymer was precipitated with 100% trichloroacetic acid (TCA) washed with 20% TCA and acetone, lyophilized, and resuspended in, and dialyzed against 50 mM imidazole (pH 7.2). Protein phosphatase assays were done as described (22). Dephosphorylation of [^{3}H]inositol 1,3,4,5-tetrakisphosphate (NEN) was performed as described by using 1 μg of the relevant glutathione *S*-transferase (GST) fusion proteins (23).

Akt Kinase Assays. U2-OS cells were transfected with plasmids encoding T7-Akt-1 and pSG5L, pSG5L-HA-PTEN, or mutant derivatives. Thirty-six hours after transfection T7 immunoprecipitates were prepared from cell lysates, collected on protein A-Sepharose and incubated in a reaction mixture containing 30 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT, 20 μM ATP, 10 μCi of [γ-^{32}P]ATP, and 5 μg of a GST fusion protein containing an Akt peptide substrate, for 30 min at 25°C. Radiolabeled substrate was separated from unincorporated [γ-^{32}P]ATP by gel electrophoresis and detected by autoradiography.

RESULTS

PTEN Induces a Block in the G_1 Phase of the Cell Cycle. Attempts at stable expression of PTEN in PTEN-null 786-O renal carcinoma and A172 glioblastoma cell lines failed to yield clonal lines that produced detectable HA-PTEN (data not shown). Next, transient assays were used to determine whether PTEN might be capable of altering cell-cycle progression. 786-O renal carcinoma cells, which lack PTEN protein (Fig. 1*D*), were transiently transfected with either empty vector or plasmids encoding either HA-tagged PTEN (PTEN;WT) or a tumor-derived PTEN catalytic domain mutant (PTEN;G129R) (4), along with a plasmid encoding the cell surface marker CD19 (pCD19). After 40 h, the DNA content of the successfully transfected cells was determined by staining cells with fluorescein isothiocyanate-conjugated anti-CD19 and propidium iodide followed by fluorescence-activated cell sorting. Wild-type PTEN reproducibly induced an increase in the percentage of cells in G_1 when compared with the vector alone or to PTEN;G129R (Fig. 1*A*). In contrast, reintroduction of plasmids encoding either the tumor suppressor proteins pRB or VHL [which is defective in 786-O cells (30)] or the dual-specificity phosphatase cdc25C failed to induce a G_1 arrest in these cells (Fig. 1*A* and data not shown). Production of HA-PTEN in two cell lines that retain endogenous PTEN protein (SAOS-2 and ACHN) did not alter the cell-cycle distribution of these cells (Fig. 1 *B*–D), but, as a positive control in pRB-null SAOS-2 cells, reintroduction of a plasmid encoding pRB did effect a G_1 arrest (Fig. 1*B*). Under these same experimental conditions, production of PTEN protein in 786-O cells did not lead to an increase in the percentage of cells harboring a sub-2*N* DNA content, suggesting that PTEN did not induce apoptosis in these cells (Table 1). Thus, PTEN specifically induced a G_1 block in 786-O cells, which lack PTEN.

Tumor-Derived Mutants Inactivate PTEN Phosphatase Activity and Cell-Cycle Control. A number of tumor-derived PTEN mutations have been reported that lie outside of the predicted phosphatase and tensin–auxilin homology domains. Three such tumor-derived mutants, PTEN;1–274, PTEN;1–336, and PTEN;Δ274–342 were tested and were defective in the cell-cycle assay (Fig. 2*A*). With the exception of PTEN;1–

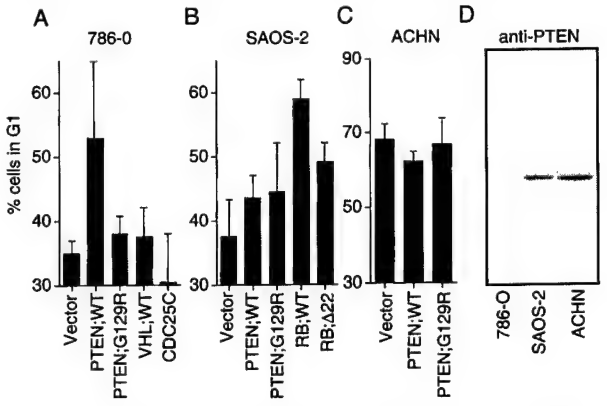

FIG. 1. PTEN induces a G_1 block. (*A*) PTEN, but not pVHL or cdc25C, induced a G_1 block in 786-O cells. 786-O cells were transiently cotransfected with a plasmid encoding CD19 (pCD19) along with plasmids encoding the indicated proteins. Forty hours after transfection, cells were fixed and the cell-cycle distribution of the successfully transfected cells was determined by fluorescence-activated cell sorting analysis. The mean and SEM of two experiments are shown. (*B*) PTEN does not alter the cell-cycle profile of SAOS-2(RB−/−) cells. SAOS-2 cells were transiently transfected with pCD19 and the plasmids encoding the indicated proteins and analyzed as in Fig. 2*A*. The mean and SEM of two experiments are shown. (*C*) PTEN does not alter the cell-cycle profile of ACHN cells. ACHN cells were transiently transfected with pCD19 or plasmids encoding the indicated proteins and analyzed as in *A*. The mean and SEM of two experiments are shown. (*D*) Immunoblot detection of PTEN protein in 786-O, SAOS-2, and ACHN cells. C54 anti-PTEN antiserum was used to detect PTEN by immunoblot analysis of protein extracts from the indicated cell lines.

Table 1. Percentage of CD19+ 786-O cells with sub-2*N* DNA content

Exp.	Vector	PTEN;WT	PTEN;G129R
1	4.6	5.1	5.8
2	2.4	1.5	1.4
3	1.0	1.3	1.0

786-O cells were transiently transfected with pCD19 and the indicated pSGL-HA expression plasmids. After 36 h, cells were harvested and processed as in Fig. 1*A*.

274, these mutant proteins were produced to levels similar to that of wild-type PTEN in 786-O cells (Fig. 2*B*). Two biochemical properties have been ascribed to PTEN. PTEN can dephosphorylate certain protein substrates containing either phosphotyrosine or phosphothreonine (33). In addition, PTEN can dephosphorylate PtdIns-3,4,5-P_3 (23). We next asked whether the three tumor-derived mutants were defective for either of these functions. When produced as GST fusion proteins, all three mutant proteins were defective in catalyzing the release of phosphate from either a ^{33}P-labeled poly(Glu_4-Tyr_1) substrate or [^{3}H]inositol 1,3,4,5-tetrakisphosphate ([^{3}H]IP_4) (Fig. 2 *C* and *D*).

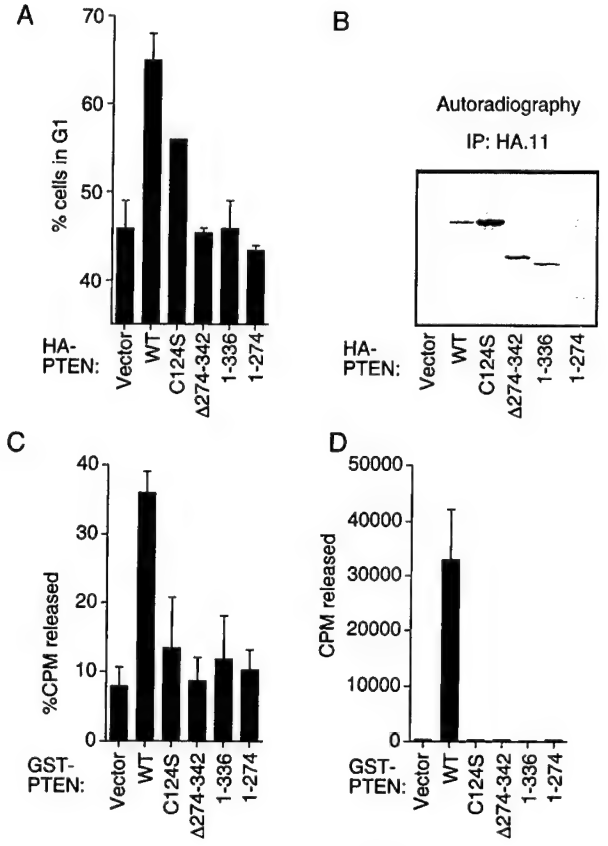

FIG. 2. Substrate trapping variants of PTEN induce a G_1 block but tumor-derived mutants do not. (*A*) Comparison of wild-type, substrate-trapping and C-terminal mutant forms of PTEN in the cell-cycle assay. 786-O cells were cotransfected with pCD19 and plasmids encoding the indicated proteins and analyzed as in Fig. 1*A*. (*B*) Expression of PTEN proteins in 786-O cells. 786-O cells were transfected with plasmids encoding the indicated proteins. Forty hours after transfection anti-HA immunoprecipitates of protein extracts prepared from metabolically labeled cells were separated by gel electrophoresis and subjected to fluorography. (*C*) Inositol phosphatase activity of GST-PTEN and mutant derivatives. The indicated GST-PTEN fusion proteins were used to dephosphorylate [^{3}H]IP_4. (*D*) Protein tyrosine phosphatase activity of GST-PTEN and mutant derivatives. The indicated GST-PTEN fusion proteins were used to dephosphorylate ^{33}P-labeled poly(Glu_4-Tyr_1) copolymers. (*A*, *C*, and *D*) The mean and SEM of two experiments are shown.

Two catalytically inert PTEN variants were created (PTEN;C124S and PTEN;D92A). Mutations of the corresponding residues in other phosphatases results in loss of enzymatic activity but allows for preservation of substrate binding and have been termed "substrate trapping" (34). As predicted, and as previously published (22, 23), the PTEN substrate-trapping mutants lack catalytic activity (Fig. 2 *C* and *D* and data not shown). Nonetheless, both PTEN;C124S and PTEN;D92A retained a partial ability to induce cells to accumulate in G_1 (Fig. 2*A* and data not shown). These data suggest that sequestration of phosphorylated substrates might be sufficient for cell-cycle inhibition by PTEN. PTEN;C124S can induce an accumulation of PtdIns-3,4,5-P_3 in cells, suggesting that there may be stable binding to this substrate (23). On the other hand the C124S mutant is defective in regulating cell motility (26). Thus, these data raised the possibility that the ability of PTEN to induce a G_1 block was distinct from cell motility control and suggested a relationship between the ability of PTEN to interact with a PtdIns substrate and the ability to arrest cells in G_1.

Protein Phosphatase Activity Is Not Sufficient for Induction of a G_1 Block by PTEN. We next compared the G129R mutant to a second mutant in which the same codon is affected (G129E). G129E is encoded by a *PTEN* allele found in the germ-line *PTEN* gene of two families afflicted with CD (35). Others have found that protein phosphatase activity of PTEN;G129E is equivalent to the wild-type protein (22) and that PTEN;G129E is comparable to wild-type PTEN in its ability to inhibit cell spreading (26). Indeed, in our assays we likewise see retention of protein phosphatase activity (Fig. 3*D*). In five preparations of GST-PTEN;G129E, activity varied from 30% to 70% of the wild-type activity (Fig. 3*D* and data not shown). However, when produced in 786-O cells, this PTEN mutant did not induce a G_1 block (Fig. 3*A*). Thus, both G129E and G129R failed to induce a G_1 block, even though the former retains protein phosphatase activity (Fig. 3*D*). We next asked whether the G129E mutant might be defective in dephosphorylating [^{3}H]IP_4, as a measure of lipid phosphatase activity. Indeed, although PTEN;WT catalyzed the dephosphorylation of [^{3}H]IP_4, neither G129R nor G129E had measurable activity in this assay (Fig. 3*C*). Thus, the ability of PTEN to induce a cell-cycle block correlated best with its ability to dephosphorylate a lipid substrate, and preservation

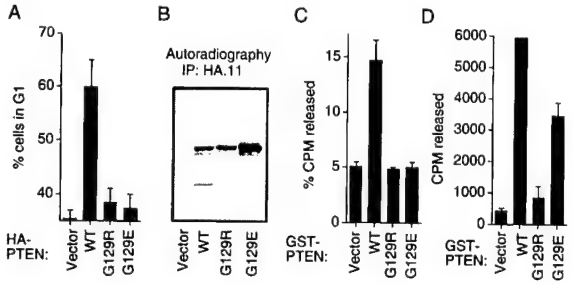

FIG. 3. CD mutant G129E preserves protein phosphatase activity but lacks inositol phosphatase activity and is incapable of inducing a G_1 block. (*A*) Comparison of wild-type PTEN and the mutants G129R and G129E in the cell-cycle assay. 786-O cells were cotransfected with pCD19 and plasmids encoding the indicated proteins and analyzed as in Fig. 1*A*. (*B*) Comparison of the expression of wild-type PTEN and the mutants G129R and G129E in 786-O cells. 786-O cells were transfected with plasmids encoding the indicated proteins. Anti-HA immunoprecipitates of protein extracts prepared from metabolically labeled cells were separated by gel electrophoresis and subject to fluorography. (*C*) Inositol phosphatase activity of GST-PTEN and mutant derivatives. The indicated GST-PTEN fusion proteins were analyzed as in Fig. 2*C*. (*D*) Protein tyrosine phosphatase activity of GST-PTEN and mutant derivatives. The indicated GST-PTEN fusion proteins were analyzed as in Fig. 2*D*. (*A*, *C*, and *D*) The mean and SEM of two experiments are shown.

of protein phosphatase activity, as measured *in vitro*, was not sufficient for the induction of a G_1 block.

Akt Is Downstream of PTEN. Thus, our data suggested that the ability of PTEN to regulate cell-cycle progression was dependent upon its ability to dephosphorylate PtdIns-3,4,5-P_3 and raised the possibility that the regulation of downstream targets of phosphatidylinositol 3-kinase might be critical for PTEN-mediated cell-cycle control. One such effector is the protein product of the protooncogene *AKT*. We next sought to determine whether PTEN might down-regulate Akt kinase activity. U2-OS cells were transiently transfected with an empty vector plasmid or a plasmid encoding T7-epitope tagged Akt along with the backbone vector plasmid or plasmids encoding HA-PTEN or PTEN;G129E. Akt was recovered by anti-T7 immunoprecipitation and used to phosphorylate a polypeptide substrate in the presence of [γ-^{32}P]ATP. PTEN efficiently down-regulated Akt kinase activity, whereas PTEN;G129E did not (Fig. 4 *A* and *B*). Identical results were obtained when this experiment was carried out in 786-O cells, which lack PTEN protein (data not shown). Thus, PTEN can negatively regulate Akt kinase activity.

We next asked whether Akt or a myristoylated form of Akt could override a PTEN-induced G_1 block. 786-O cells were transiently transfected with pCD19 and plasmids encoding either empty vector or wild-type PTEN and either empty pLNCX vector or pLNCX plasmids encoding Akt or the indicated derivatives. Although wild-type Akt had a minimal effect on the PTEN-induced G_1 block, a myristoylated form of Akt that is targeted to the membrane independently of PtdIns-3,4,5-P_3 overcame a PTEN block. In contrast, kinase-inactive versions of both Akt and Myr-Akt were unable to override PTEN (Fig. 4*C*). PTEN levels were unchanged by overproduction of Akt or the indicated derivatives (data not shown). These data suggest that PTEN-mediated cell-cycle inhibition depends on negative regulation of the phosphatidylinositol 3-kinase/Akt signaling pathway.

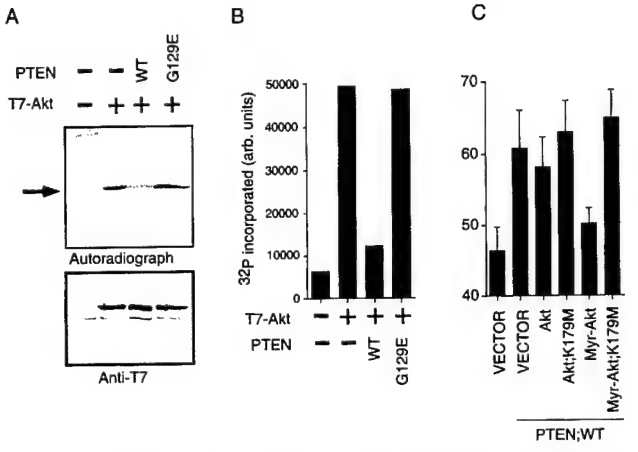

FIG. 4. PTEN inhibits Akt kinase activity and an activated form of Akt can override a PTEN induced G_1 block. (*A*) Inhibition of Akt kinase by wild-type PTEN. U2-OS cells were transfected with plasmids encoding the indicated proteins. After transfection, anti-T7 immunoprecipitates were prepared and used to phosphorylate a GST-peptide substrate *in vitro*. Autoradiography (*Upper*) and anti-T7 immunoblot (*Lower*) of the same membrane are shown. The black arrow indicates the position of the substrate. Results are representative of two experiments. (*B*) Quantitation of ^{32}P incorporation in *A* with a PhosphoImager. (*C*) Myr-Akt overrides a PTEN-induced G_1 block. 786-O cells were transiently cotransfected with pCD19 and plasmids encoding PTEN with empty vector (pLNCX) or with plasmids encoding the indicated Akt proteins. After transfection the cells were analyzed as in Fig. 1*A*. The mean and SEM of two experiments are shown.

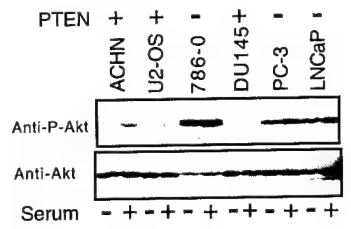

FIG. 5. Cells lacking PTEN protein contain high levels of Akt, phosphorylated on Ser-473. The indicated cells lines were serum-deprived for 24 h in duplicate. One p100 plate from each pair was refed with fresh medium containing 10% fetal calf serum for 90 min, after which protein extracts were prepared and subject to immunoblot analysis with an antibody directed at Akt phospho-Ser-473 (*Upper*) or an antibody that recognizes Akt independent of its phosphorylation status (*Lower*).

If Akt is a critical downstream target of PTEN tumor suppressor function, tumors or cell lines that lack PTEN protein might be predicted to harbor elevated levels of Akt kinase activity. As a measure of endogenous Akt activity, we subjected cell extracts to immunoblotting with an antibody specific to phospho-Ser-473 on Akt. Phosphorylation of this residue along with Thr-308 is required for Akt kinase activity (36). Protein extracts from duplicate plates of a panel of either PTEN+ or PTEN− cells were prepared after serum deprivation or after 90 min of serum stimulation and immunoblotted with the phospho-specific antibody. Protein extracts prepared from cells that contain PTEN protein by immunoblot, ACHN, DU145, and U2-OS (Fig. 1*D* and data not shown) were found to have little phosphorylated Akt (Fig. 5 *Upper*). In contrast, cells that lack PTEN protein, 786-O, LNCaP, and PC-3 (Fig. 1*D* and data not shown), had increased levels of phosphorylated Akt that was not down-regulated by serum withdrawal (Fig. 5 *Upper*). Levels of total Akt-1 protein in these cells were comparable (Fig. 5 *Lower*). Thus, loss of PTEN, in these tumor cell lines, was specifically correlated with a deregulation of the phosphorylated form of Akt.

DISCUSSION

Reintroduction of PTEN into 786-O cells led to an increase in the proportion of cells in G_1. Whether this represents a block in G_1 or a prolongation of G_1 awaits further study. Every human derived PTEN mutation we have tested to date has been defective in this cell-cycle assay. Thus, it is clear that the G_1 arrest is a reflection of a critical function of PTEN that is tightly linked to its function as a tumor suppressor protein.

What are the underlying mechanisms by which PTEN might exert control over progression through G_1? First, this function appears to be quite distinct from PTEN-mediated inhibition of cell spreading and cell motility. PTEN inhibition of cell motility and adhesion was observed in cells containing endogenous PTEN, was observed in cells only when grown on a fibronectin matrix, and was not observed with the PTEN;C124S mutant. In contrast, PTEN overexpression induced a G_1 block in cells lacking, but not in cells producing, PTEN protein, could be induced in cells plated on poly-(L-lysine)-coated plates, and was partially induced by the C124S mutant. Finally, PTEN;G129E clearly separates these two functions, because this mutant inhibits cell spreading comparably to the wild-type protein (26) but is incapable of arresting 786-O cells in G_1 (Fig. 3). Thus, these data suggest that the alterations in cell-cycle profile are not an indirect consequence of inhibition of cellular adhesion. Furthermore, that PTEN;G129E preserves protein phosphatase activity and can negatively regulate cell spreading yet is linked to the development of CD suggests that these functions are not sufficient for suppression of the CD phenotype. On the other hand, PTEN;G129E is defective in the cell-cycle assay. Thus, this

biological assay might be indicative of PTEN interactions with critical physiological substrates, regulation of which might be essential for preventing the onset of CD. Indeed, this mutant is defective in catalyzing the dephosphorylation of $[^3H]IP_4$, suggesting that *in vivo* dephosphorylation of PtdIns-3,4,5-P_3 is a critical requirement for PTEN-mediated suppression of CD. Similar results were recently reported by two groups (37, 38). Thus, we propose that the development of CD is linked to a loss of PTEN lipid phosphatase activity and to the subsequent deregulation of the cell cycle.

In support of this notion, mice that have only a single intact *PTEN* allele develop a syndrome not unlike CD and are phenotypically characterized by hyperplasia and dysplasia of the skin, gastrointestinal tract, and prostate (27). Notably, in the prostate of *PTEN*+/− mice both Ki-67 staining and the mitotic index indicated a significant increase in the proportion of cells that were proliferating when compared with wild-type mice. These data indicate that in murine prostate epithelial cells, the cell-cycle distribution has been altered by PTEN loss (27). In addition, wide-spread increased bromodeoxyuridine incorporation is found in PTEN mutant embryos at day 7.5 to 8.5 when compared with wild-type embryos (39). Finally, PTEN-mediated growth inhibition was recently linked to induction of a G_1 arrest rather than induction of apoptosis in glioblastoma cell lines (37).

Thus, our data and the data describing proliferative abnormalities in PTEN+/− mice suggest that PTEN plays a role in cell-cycle control. This function in turn appears to require lipid phosphatase activity. These data led us to ask whether Akt, a known downstream target of phosphatidylinositol 3-kinase that is activated by PtdIns-3,4,5-P_3, could act downstream of PTEN. First, we found that wild type but not the G129E mutant of PTEN could inhibit Akt kinase activity, suggesting again that PTEN protein phosphatase activity is not sufficient for inhibition of Akt. We next asked whether Akt could override a PTEN-mediated cell-cycle block. In keeping with the role of PTEN in limiting the availability of PtdIns-3,4,5-P_3, wild-type Akt was ineffective in this assay, whereas expression of a myristoylated form of Akt led to an override of the PTEN cell-cycle block. These data support the notion that Akt is an important downstream target of PTEN regulation. Similar conclusions have been reached by studying the effect of PTEN loss on murine fibroblasts (39).

Inducible expression of phosphatidylinositol 3-kinase can induce DNA synthesis in the absence of serum and is thus sufficient for initiation of this process (40). Akt and phosphatidylinositol 3-kinase can activate a number of downstream targets that may be involved in the regulation cell proliferation (41). The ribosomal protein $p70^{S6K}$ regulates the increased translation of a subset of mRNA species thought to be important for cell-cycle progression and, indeed, inactivation of $p70^{S6K}$ function leads to an arrest of cells in G_1 (42, 43). Likewise, Akt can induce phosphorylation of 4E-BP1, an event that leads to 4E-BP1 dissociation from the eukaryotic initiation factor eIF4E and a subsequent "disinhibition" of translation (44). eIF4E when overproduced transforms NIH 3T3 cells and, thus, like Akt, is an attractive tumor suppressor target (45). Which component(s) in the phosphatidylinositol 3-kinase/Akt pathway are rate-limiting for S phase entry is not known.

Akt is also an important component of a cell-survival signaling pathway and in this capacity can phosphorylate and inactivate the Bcl-2 family member BAD, rendering it incapable of blocking Bcl-2 or Bcl-X_L activity (41, 46, 47). Recent data has shown that PTEN−/− cells are resistant to apoptotic stimuli and that regulation of Akt is abnormal in PTEN-deficient fibroblasts (39). To date, we have not seen an increase in apoptosis upon reintroduction of PTEN into 786-O cells (Table 1). Among many possibilities, these data may indicate that additional inactivating mutations have been sustained in this cell line, rendering it incapable of responding to a variety of apoptotic signals. Finally, increases in phosphatidylinositol 3-kinase activity do not uniformly result in protection from apoptosis. In certain circumstances, phosphatidylinositol 3-kinase activity can induce cell-cycle progression and, in fact, promote apoptosis (40) (R. Narsimhan and T.M.R., unpublished data). The notion that PTEN may regulate both cell-cycle and cell-survival functions is strikingly similar to the tumor suppressor functions imputed to p53. Thus PTEN, like p53, may serve to coordinate these activities, although possibly in response to different sets of signals (48).

If Akt is a critical downstream target of PTEN, tumor cells that lack PTEN might be predicted to harbor excessive Akt activity. Immunoblots of protein extracts from cells that were characterized as PTEN− or PTEN+ (Fig. 1*D* and data not shown) were found to have a marked increase in the amount of Akt phosphorylated on Ser-473 (Fig. 5). This inverse correlation between PTEN and activated Akt again argues for a role for PTEN in Akt regulation in tumors. In this regard, inhibition of Akt activity by a dominant negative form of Akt blocks BCR-ABL transformation of murine myeloid cells and stable expression of antisense Akt2 reduces tumorigenicity of pancreatic cancer cell lines known to have amplified Akt (49, 50). Thus, inhibition of Akt family members in these settings does demonstrate antitumor activity. Whether activated Akt is necessary for the transformed phenotype of PTEN null tumors is a critical question. Nonetheless, our data raise the possibility that PTEN null tumors may be susceptible to Akt inhibition as a cancer treatment strategy.

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Loss of PTEN Expression in Paraffin-embedded Primary Prostate Cancer Correlates with High Gleason Score and Advanced Stage[1]

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ABSTRACT

The tumor suppressor gene PTEN/MMAC-1/TEP-1 (referred to hereafter as PTEN) maps to chromosome 10q23 and encodes a dual specificity phosphatase. The PTEN protein negatively regulates cell migration and cell survival and induces a G_1 cell cycle block via negative regulation of the phosphatidylinositol 3′-kinase/protein kinase B/Akt signaling pathway. *PTEN* is frequently mutated or deleted in both prostate cancer cell lines and primary prostate cancers. A murine polyclonal antiserum was raised against a glutathione *S*-transferase fusion polypeptide of the COOH terminus of PTEN. Archival paraffin tissue sections from 109 cases of resected prostate cancer were immunostained with the antiserum, using DU145 and PC-3 cells as positive and negative controls, respectively. PTEN expression was seen in the secretory cells. Cases were considered positive when granular cytoplasmic staining was seen in all tumor cells, mixed when areas of both positive and negative tumor cell clones were seen, and negative when adjacent benign prostate tissue but not tumor tissue showed positive staining. Seventeen cases (15.6%) of prostate cancer were positive, 70 cases (64.2%) were mixed, and 22 cases (20.2%) were negative. Total absence of PTEN expression correlated with the Gleason score ($P = 0.0081$) and correlated more significantly with a Gleason score of 7 or higher ($P = 0.0004$) and with advanced pathological stage (American Joint Committee on Cancer stages T3b and T4; $P = 0.0078$). Thus, loss of PTEN protein is correlated with pathological markers of poor prognosis in prostate cancer.

INTRODUCTION

PTEN is a tumor suppressor gene that maps to the 10q23.3 interval (1–3). The protein product, PTEN, shares homology with the cytoskeletal protein tensin and the secretory vesicle protein auxilin and also with dual specificity phosphatases. Indeed, recombinant PTEN exhibits activity against both phosphotyrosine- and phosphothreonine-containing protein substrates (4). Overexpression of PTEN suppresses tumor colony formation in certain cell lines and can suppress tumor formation in nude mice (5–7). PTEN overexpression can also negatively regulate cellular adhesion and cell mobility on fibronectin-coated plates (8). This activity may result from PTEN-mediated dephosphorylation of focal adhesion kinase. PTEN may also alter mitogen-activated protein kinase signaling (9).

PTEN can also act as a lipid phosphatase. Specifically, PTEN can dephosphorylate phosphotidylinositol 3,4,5-trisphosphate and phosphotidylinositol 3,4-trisphosphate, which are both direct products of PI3K[3] activity (10). We and others have shown that PTEN can inhibit cell cycle progression and induce a G_1 arrest. This function appears to require the lipid phosphatase activity of PTEN, resulting in the negative regulation of the PI3K/Akt signaling pathway (11–13). A significant increase in the level of the cell cycle kinase inhibitor $p27^{KIP1}$ occurs with concomitant decreases in G_1 cyclin-dependent kinase activity upon the introduction of PTEN into human glioblastoma U87MG cells, suggesting that p27 may be a target of the PTEN cell cycle arrest pathway (13). In keeping with these data, heterozygous loss of the murine *PTEN* gene (*mPTEN*) leads to an increase in the mitotic index and the Ki-67 staining index in the murine prostate (7). In addition, PTEN negatively regulates Akt-dependent cell survival (14–17). Akt is one of the key regulatory molecules involved in the protection of cells against apoptosis. These data support the idea that PTEN negatively regulates cell growth and/or proliferation through its ability to act as an *in vivo* phosphoinositide 3-phosphatase, thus negatively regulating the PI3K/Akt signaling pathway.

Germ-line mutations of *PTEN* have been detected in cases of Cowden disease and Bannayan-Zonana syndrome, two related hamartoma syndromes (18–20). Patients with Cowden disease have an elevated risk of various cancers, including breast and thyroid cancer. Alterations of the second *PTEN* allele have been demonstrated in gastrointestinal polyps in patients with Cowden disease (21).

Somatic alterations of *PTEN* are common in certain cell lines and in primary tumors including gliomas (22–25), melanoma (26, 27), and thyroid (28) and endometrial cancers (29, 30). On the other hand, somatic alterations are rare in breast (31) and renal cancer (32) and were not detected in a series of squamous carcinomas from the head and neck (33). *PTEN* mutations and allele loss at 10q23 appear to be a late event in glioblastoma, melanoma, and prostate cancer (22–26, 34). In contrast, *PTEN* alterations are more common in benign tumors than in malignant thyroid tumors (28) and also occur in a proportion of cases of endometrial hyperplasia, a precursor of endometrial carcinoma (35), suggesting that the genetic alteration may occur at an early stage in these tumors.

Prostate cancer is the most prevalent form of cancer in men in the Western world and is the second most common cause of male cancer deaths in the United States (36). Pathological stage and Gleason grade are important predictors of prognosis in patients with primary prostate cancer who undergo radical prostatectomy. Prostate cancer, however, is a remarkably heterogeneous disease. Distinguishing tumors associated with a poor outcome at the time of radical prostatectomy is problematic. The molecular mechanisms of prostate carcinogenesis remain poorly understood. LOH of 10q has been reported to occur in prostate cancer with a high frequency (30–60%; Refs. 37 and 38), and two distinct, commonly deleted regions have been identified at 10q22–q24 and 10q25, respectively, implying the presence of putative tumor suppressor genes at these loci (38). Homozygous deletions and somatic mutations of *PTEN* have been identified in prostate cell lines and tumor specimens (1, 2, 34, 39–45). Marked heterogeneity of *PTEN* alterations has been observed in metastatic prostate cancer tissues (43). Loss of PTEN expression is more frequently detected in xenografts of cell lines (34). *PTEN* may be inactivated by mechanisms other than gene deletion and mutations, including promoter methylation or translational modification (34). However, other groups failed

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[3] The abbreviations used are: PI3K, phosphatidylinositol 3′-kinase; LOH, loss of heterozygosity.; GST, glutathione *S*-transferase; HA, hemagglutinin; PIN, prostatic intraepithelial neoplasia.

to detect *PTEN* methylation in prostate, bladder, and renal cell cancer with LOH of 10q when a PCR-based assay was used (32, 40).

In this study, we wanted to assess the extent of loss of the PTEN protein in prostate cancer using immunohistochemistry. We analyzed the pattern of immunohistochemical staining in 109 cases of paraffin-embedded resected prostate cancer using a murine polyclonal antibody to PTEN. Detection of PTEN protein was correlated with the Gleason score and the pathological stage of the tumor, known prognosticators in prostate cancer.

MATERIALS AND METHODS

Tissue Specimens. We used cases from a prostate database consisting of 128 paraffin-embedded prostate cancers that had been collected in the Department of Pathology, Beth Israel Deaconess Medical Center, West Campus, dating from 1990–1997. This database has been described previously (46–49). Nineteen cases were transurethral resection specimens. We used the remaining 109 radical prostatectomy specimens for this study. Follow-up data were available on 69 cases in the database, with a mean patient follow-up of 19.84 months. The pathological tumor (T) stage (American Joint Committee on Cancer; Ref. 50) and Gleason score were available in each case. Five of the 109 cases had been treated with preoperative total androgen ablation. For each case, a representative paraffin block was selected that contained both tumor and benign prostate tissue.

Processing of Cell Lines and Cell Blocks. DU145, PC-3, and LNCaP prostate cancer cell lines were obtained from the American Type Culture Collection. The U2-OS cell line was a generous gift of W. G. Kaelin (Dana-Farber Cancer Institute). DU145 cells contain one wild-type *PTEN* allele and a second variant allele (*M134L*). PC-3 cells have sustained a homozygous deletion of *PTEN*. LNCaP cells have a deletion of one allele and a mutation of the other *PTEN* allele, and the genetic state of *PTEN* has not been characterized in U2-OS cells. LNCaP and PC-3 cells were grown in RPMI 1640 supplemented with D-glucose, HEPES buffer, L-glutamine, PP_i, penicillin, streptomycin, and 10% fetal bovine serum. DU145 cells were maintained in DMEM supplemented with penicillin, streptomycin, and 10% fetal bovine serum, and U2-OS cells were maintained in DMEM supplemented with penicillin, streptomycin, and 10% fetal clone (HyClone). All cells were grown on P100 tissue culture dishes at 37°C. LNCaP and PC-3 cells were grown in a 5% CO_2 atmosphere, and DU145 and U2-OS cells were grown in a 10% CO_2 atmosphere. Cell pellets were created from DU145 and PC-3 cells, fixed in 10% formalin overnight, and then processed in the regular manner for pathology specimens to produce paraffin cell blocks.

Plasmids. A cDNA fragment of the *PTEN* gene encoding amino residues 239–403 was amplified by PCR using primers WRSO-56 (5′-GACTGGATCCATGTACTTTGAGTTCCCTCAGCC-3′) and WRSO-57 (5′-CGCGGAATTCTCAGACTTTTGTAATTTGTGTATGC-3′) from a cDNA library derived from human embryonic kidney 293 cells (51). The resulting PCR fragment was isolated, restricted with *Bam*HI and *Eco*RI, and ligated to similarly restricted pSG5L to produce pSG5L-PTEN (239–403). This cDNA was confirmed by sequencing. The insert from this plasmid was excised and ligated to *Bam*HI/*Eco*RI-restricted pGEX2T vector to produce pGEX2T-PTEN (239–403) plasmid.

Antibodies. Recombinant GST-PTEN (239–403) was produced in *Escherichia coli* and affinity-purified on glutathione-Sepharose beads by conventional methods (52). Mice were inoculated with 100 μg of GST-PTEN (239–403) mixed with Freund's complete adjuvant. Two weeks later, the mice received a subsequent boost of 100 μg of the purified protein in Freund's incomplete adjuvant. Immune sera (M1) was obtained by orbital sinus puncture.

***In Vitro* Translation, Immunoprecipitation, and Immunoblotting.** Full-length HA-tagged PTEN protein (HA-PTEN) was produced *in vitro* by coupled transcription and translation of the pSG5L-PTEN plasmid using the TnT kit (Promega, Madison, WI). Cell extracts were prepared in the following manner. Cells grown on P100 plates were washed twice with PBS and then lysed on the plate in 500 μl of TNN buffer [150 mM NaCl, 50 mM Tris (pH 7.4), and 0.5% NP40] at 4°C for 20 min. Collected extracts were then cleared by centrifugation at 14,000 rpm for 15 min. Immunoprecipitations of *in vitro* translated products were carried out at 4°C in NET-N buffer [120 mM NaCl, 10 mM EDTA (pH 8.0), 100 M Tris (pH 7.4), and 0.5% NP40] along with 5 μl of *in vitro* translated PTEN in 250 μl of NET-N. One μl of antiserum was used per immunoprecipitation experiment. Immune complexes were captured on protein A-Sepharose beads (30 μl of 1:1 beads), washed five times with NET-N, and boiled in 1× Laemmli sample buffer. Whole cell extracts or immunoprecipitates were separated by vertical gel electrophoresis on 7.5% gels. Proteins were transferred to Sequi-blot polyvinylidene difluoride membrane (Bio-Rad) by wet transfer in Towbin's buffer for 6–16 h. Immunoblots were blocked in TBS + 4% milk. M1 was used at a concentration of 1:10,000 in TBS + 4% milk. Alkaline phosphatase-conjugated goat antimouse antibody was the secondary antibody.

Immunohistochemistry. Five-μm sections were cut from the selected paraffin blocks of prostate tumor and the DU145 and PC-3 cell blocks, mounted on charged glass slides, baked at 60°C for 60 min, deparaffinized, and rehydrated through graded alcohol rinses. Slides were immersed in 10 mM/liter citrate buffer (pH 6.0; Biogenex, San Ramon, CA) and microwaved in a 750 W oven inside a pressure cooker for 30 min. The slides were cooled at room temperature for 15 min and rinsed in tap water. A 1:2000 dilution of M1, the PTEN murine polyclonal antiserum, was applied for 32 min at 37°C. An automated processor (Ventana ES; Ventana Medical Systems, Tuscon, AZ) was used to incubate the slides in blocker (10% normal goat serum and 10% normal horse serum in Ventana diluent) for 8 min, followed by an incubation in secondary antibody conjugated to an avidin-biotin peroxidase complex (antirabbit and antimouse). Finally, 3,3′-diaminobenzidine was used as a substrate to detect bound antibody complex. The slides were counterstained with hematoxylin. Standardization of the incubation and development times allowed an accurate comparison of expression levels in all cases.

Analysis of Immunohistochemical Staining. Positive cases were defined by the presence of granular, crisp cytoplasmic staining, as seen in the DU145 positive control samples. The cases were initially divided into three groups: (*a*) positive (the entire tumor showed staining); (*b*) mixed (both positive and negative cells/glands were present); and (*c*) negative (no staining was seen in the represented tumor). The grading of PTEN expression was performed without knowledge of the Gleason score or pathological stage. The presence of positive staining in PIN was noted. The cases were then divided into those that showed positive staining (positive and mixed groups) and those with a total absence of staining (negative group).

Statistical Analysis. We tested for associations between PTEN expression and Gleason score or pathological stage of disease using the Mann-Whitney nonparametric *U* test, the c^2 test, or Fisher's exact test, as appropriate. All calculations were performed using StatView 4.5 software (Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Characterization of the Antibody. A murine polyclonal antiserum (M1) was raised against a protein chimera encoding GST and PTEN amino acid residues 237–403. HA-PTEN was produced by *in vitro* translation and subjected to immunoprecipitation with M1. Purified anti-HA antibody and the M1 preimmune sera served as positive and negative controls, respectively. Both the M1 antiserum and the anti-HA antibody immunoprecipitated HA-PTEN, whereas the nonimmune serum did not (Fig. 1, *left panel*). To determine whether M1 might specifically recognize the endogenous PTEN protein, whole cell protein extracts were prepared from U2-OS osteosarcoma cells and DU145 and PC-3 prostate carcinoma cells, separated by electrophoresis, and subjected to immunoblotting with M1 antiserum (Fig. 1, *right panel*). M1 recognized a protein species of approximately M_r 58,000 that is present in DU145 and U2-OS cells but is absent in PC-3 cells. PC-3 cells have sustained a biallelic deletion of the *PTEN* gene, whereas DU145 contains a wild-type *PTEN* allele and an allele harboring a missense change at codon 134 (M134L). This protein species migrates slightly faster than the *in vitro* translated HA-PTEN. Taken together, these data indicate that the recognized protein is endogenous PTEN.

PTEN Expression in Human Prostate Cancer Cell Lines. Next we asked whether M1 was capable of recognizing PTEN by immu-

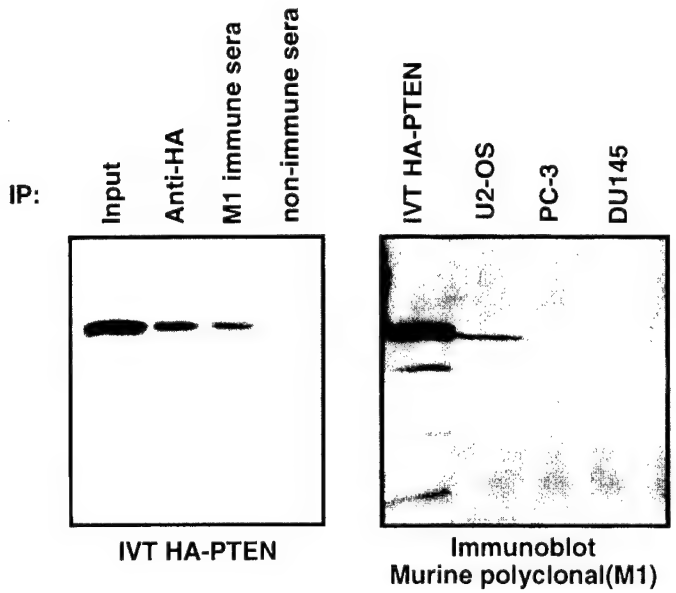

Fig. 1. Immunoprecipitation of *in vitro* translated HA-PTEN with the murine polyclonal antisera M1. HA-PTEN was produced by *in vitro* translation and immunoprecipitated with either anti-HA antibody, murine polyclonal antisera M1, or nonimmune murine antisera, as indicated (*left panel*). The input is 5 μl of *in vitro* translated HA-PTEN loaded in sample buffer. Immunoblot detection of endogenous PTEN was performed using murine polyclonal antisera M1.Whole cell extracts (150 μg) prepared from U2-OS cells, PC-3 cells, and DU145 cells (as indicated) and HA-PTEN produced by *in vitro* translation were separated by gel electrophoresis, transferred to polyvinylidene difluoride membrane, and immunoblotted with murine polyclonal antisera M1 (*right panel*).

nohistochemistry. Cell blocks were prepared from DU145 and PC-3 cells that were grown in culture. M1 was then used to detect PTEN in 5-μm sections from these paraffin blocks by immunohistochemical means. Strong positive granular cytoplasmic staining was detected in DU145 cells, but PC-3 cells were negative (Fig. 2, *A* and *B*). Thus, sections of cell blocks of DU145 and PC-3 cell lines served as positive and negative controls, respectively. Preimmune serum also served as a negative control.

Patient Databases and Tumor Characteristics. The mean patient age at the time of surgery was 65.2 ± 8.4 years, with an age range of 40–86 years. The Gleason score of the tumors ranged from 4–9 with the following frequency: (*a*) Gleason score = 4, 4 tumors; (*b*) Gleason score = 5, 9 tumors; (*c*) Gleason score = 6, 17 tumors; (*d*) Gleason score = 7, 58 tumors; (*e*) Gleason score = 8, 12 tumors; and (*f*) Gleason score = 9, 9 tumors. The median Gleason score was 7. The cases were then subdivided into two groups: (*a*) those with a Gleason score < 7 (30 cases); and (*b*) those with a Gleason score ≥ 7 (79 cases; Table 1). The cases were divided into two groups: (*a*) those with either organ-confined disease or disease extends through into the prostate capsule (T1–T3a; 83 cases); and (*b*) those with seminal vesicle involvement or metastases to the lymph nodes (T3b and T4; 26 cases; Table 2).

PTEN Expression in Human Prostate Tissue. Benign prostate epithelium showed positive staining for PTEN with granular cytoplasmic staining observed in the prostatic secretory cells. PIN was present in the selected slides in 58 cases, and all cases showed positive staining (Fig. 3, *A1* and *A2*). The cases were initially divided into three groups: (*a*) positive (the entire tumor showed staining); (*b*) mixed (both positive and negative cells/glands were present); and (*c*) negative (no staining was seen in the represented tumor). Heterogeneous staining of the tumors was present. Seventeen cases (15.6%) were positive (Fig. 3, *B1* and *B2*). Seventy cases (64.2%) showed a mixed staining pattern. Specifically, there were areas of tumor that stained positively, whereas other areas of tumor showed negative staining (Fig. 3, *C1* and *C2*). The remaining 22 tumors (20.2%) were negative (Fig. 3, *D1* and *D2*). The cases were subsequently divided into those that showed positive staining (positive and mixed groups) and those with a total absence of staining (negative group). The results of PTEN expression in each group were compared.

Correlation of PTEN Expression with Gleason Score and Pathological Stage. Loss of PTEN expression correlated significantly with increasing Gleason score ($P = 0.0081$), and when cases were divided into those with a Gleason score < 7 and those with a Gleason score ≥ 7, the correlation with a Gleason score ≥ 7 was highly significant ($P = 0.0004$). Loss of PTEN expression also correlated with advanced disease (pathological tumor stage T3c and T4; $P = 0.0078$). PTEN expression was seen in two of the five tumors where patients had undergone preoperative total androgen ablation.

Follow-up for the cohort was too short to give meaningful survival figures because only four deaths had occurred in the study group.

DISCUSSION

In this study, a murine polyclonal antiserum (M1) was raised against a protein chimera encoding GST and PTEN amino acid residues 237–403. Using this antiserum, the expression of the PTEN protein was determined by immunohistochemistry in 109 prostate cancers of varying grade and pathological stage. Whereas PTEN was expressed in all cases of PIN, the presumed precursor lesion of

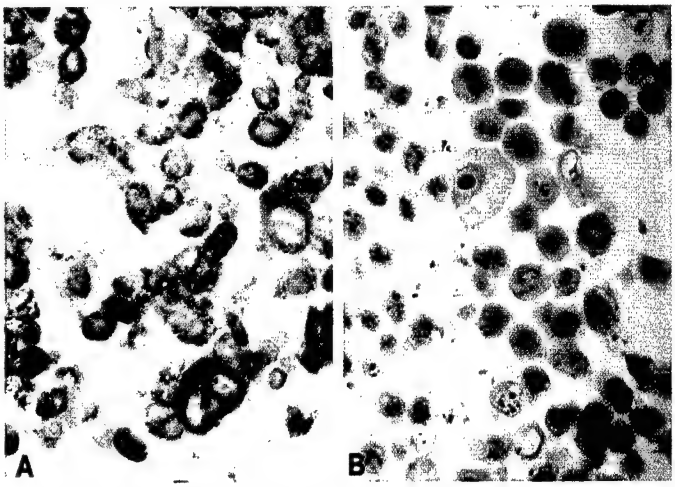

Fig. 2. PTEN expression in prostate cancer cell lines. Sections of cell blocks prepared from (*A*) DU145 cells (which contain one wild-type *PTEN* allele) or (*B*) PC-3 cells (which show a homozygous deletion of *PTEN*) were stained with M1 antiserum as described in "Materials and Methods." ×400.

Table 1 *Observed frequencies for PTEN expression and Gleason score*

	PTEN expression[a]		
Gleason score	Positive (%)	Mixed (%)	Negative (%)
<7	10 (9.2)	20 (18.3)	0 (0)
≥7	7 (6.4)	50 (45.9)	22 (20.2)

[a] Negative group *versus* positive plus mixed groups, $P = 0.0004$.

Table 2 *Observed frequencies for PTEN expression and pathological stage*

	PTEN expression[a]		
Pathological stage	Positive (%)	Mixed (%)	Negative (%)
T1–T3a	14 (12.8)	57 (52.3)	12 (11.0)
T3b and T4	3 (2.8)	13 (11.9)	10 (9.2)

[a] Negative group *versus* positive plus mixed groups, $P = 0.0078$.

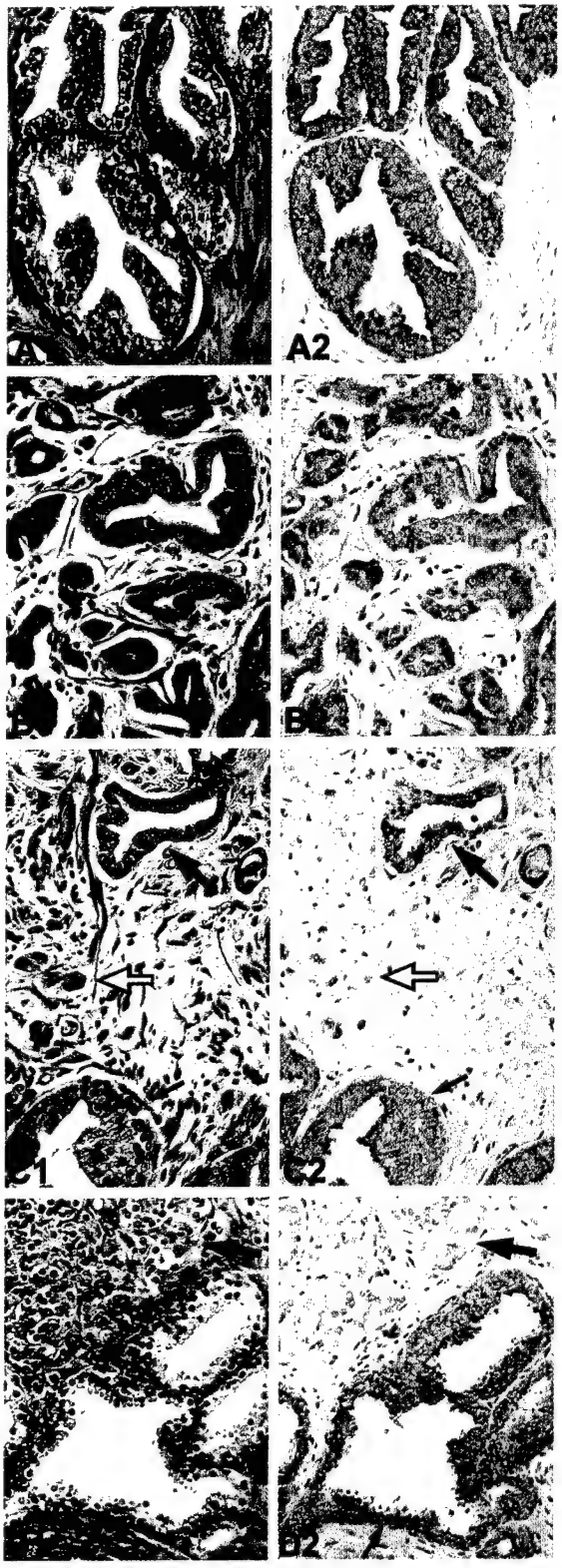

Fig. 3. PTEN expression in prostate tissue. *A*, PIN. *A1*, H&E-stained section of PIN. *A2*, positive staining for PTEN in PIN. *B*, prostate cancer (Gleason score, 3 + 3 = 6). *B1*, H&E-stained section. *B2*, positive staining for PTEN in prostate cancer. *C*, an example of heterogeneous expression of PTEN in a case of prostate cancer (Gleason score, 3 + 5 = 8). *C1*, prostate cancer. Gleason grade 3 cancer (*large black arrow*), Gleason grade 5 cancer (*open arrow*), and PIN (*short black arrow*). H&E-stained section. *C2*, Gleason grade 3 prostate cancer stains positively for PTEN (see *large black arrow*). In contrast, Gleason grade 5 prostatic carcinoma is negative for PTEN expression (*open arrow*). PIN shows positive staining for PTEN (*short black arrow*). *D*, benign prostate acinus with surrounding prostate cancer (Gleason score, 4 + 3 = 7). *D1*, H&E-stained section. *Small arrow*, benign prostatic glandular epithelium; *large arrow*, prostatic adenocarcinoma). *D2*, positive staining for PTEN in benign prostate tissue and absence of staining for PTEN in prostate cancer. ×200. All tissue sections were processed as described in "Materials and Methods."

prostate cancer (53), total loss of expression of PTEN was found in 20.2% of the prostate cancers. In an additional 70 of 109 cases (64.2%), there was a mixed pattern of staining, with areas of tumor with positive staining and other areas that were negative for PTEN. A mixed pattern of staining for PTEN was also recently found in glioma (54). Finally, 15.6% of tumors appeared to have homogenous positive staining for PTEN.

In this data set, complete PTEN loss was found to correlate significantly with the presence of high-stage disease (pathological stage T3b and T4; $P = 0.0078$). Indeed, *PTEN* mutations and allele loss at 10q23 have been reported to occur as a late event in most, albeit not all, tumors, including prostate cancers (22–26, 28, 34, 35). We also found that loss of PTEN expression correlated significantly with increasing grade of prostate cancer, *i.e.,* Gleason score ($P = 0.0081$). When cases were divided into those with a Gleason score < 7 or ≥ 7, loss of PTEN expression correlated significantly with a Gleason score ≥ 7 ($P = 0.0004$). A cut point between Gleason score 6 and 7 has previously been recommended when compression of the Gleason score is required (55). Similarly, *PTEN* is altered in high-grade gliomas, but not in low-grade gliomas (22, 23). On the other hand, *PTEN* alteration occurs in all three grades of endometrial cancer (29, 30), and mutation of a germ-line *PTEN* allele predisposes carriers to breast and thyroid cancer in humans and to a number of malignancies including prostate cancer in mice (7, 18, 20, 56). Thus, PTEN appears to play a role in the initiation of certain tumors, including a murine form of prostate cancer, and may play a role in the progression of other tumors such as gliomas and prostate cancer. Although seemingly paradoxical, the role of PTEN loss as an initiating event *versus* its role as an agent of progression might arise from fundamental differences between tissues with respect to the order of addition of various oncogenic events. For example, the human adult male prostate epithelial cell might not tolerate loss of PTEN unless the loss was first preceded by a permissive mutational event. On the other hand, this paradox, at least with respect to the prostate, might simply reflect upon our current ability, or lack thereof, to detect certain PTEN mutational events. Indeed, in our data set, the vast majority (85%) of tumors had a portion of the tumor in which PTEN staining was absent, in keeping with the marked heterogeneity of *PTEN* alterations that has been reported previously in metastatic prostate cancer samples (43). If it is the PTEN-null portion of the tumor that is destined to become the predominant metastatic clone, then the apparent lack of PTEN mutations in such organ-confined tumors might simply result from a lack of detection by conventional methodologies.

PTEN alterations have also been described in prostate cancer cell lines, xenografts, and tumors (1, 2, 34, 39–44). The true number of inactivating events is likely to be greater because the presence of sequence mutations in promoter/regulatory regions was not excluded by these studies. Of interest, there has been no evidence of *PTEN* promoter methylation in prostate cancers or bladder and renal cancers with 10q LOH using a DNA-based assay (32, 40). However, in certain prostate cancer cells, *PTEN* mRNA was restored after treatment with the demethylating agent 5-azadeoxycytidine (34). It is possible that methylation of a transcription factor for *PTEN* may play a role in the regulation of the gene.

Although we did not assess the genetic status of *PTEN* in our cases, loss of expression as assessed by immunohistochemistry might reflect a majority of the possible mechanisms resulting in PTEN inactivation. These would include direct inactivation by homozygous deletion, nonsense mutation, certain internal deletions, and promoter methylation or indirect inactivation such as loss of a PTEN-directed transcription factor or posttranscriptional modification, such as that which occurs with cdc25, another dual specificity phosphatase (57). Mis-

sense mutations, which do not grossly destabilize the protein product, would not be accounted for by immunohistochemistry.

PTEN appears to function, at least in part, by acting to brake cell cycle progression (11–13). We and others (11, 12) have previously demonstrated that this function appears to require PTEN lipid phosphatase activity, suggesting that cell cycle regulation may result from inhibition of the PI3K pathway. We further demonstrated that activated forms but not wild-type forms of the proto-oncogene Akt were capable of overriding a PTEN-mediated cell cycle block, raising the possibility that Akt might be an important downstream target of PTEN with respect to cell cycle progression (12). Similar conclusions have been reached with respect to the function of PTEN as a regulator of apoptosis or cell survival (14–17, 58). These data, taken together, suggest the possibility that targeted inhibition of the PI3K/Akt pathway might be of therapeutic value in patients with prostate cancer. We and others (12, 59) have also shown that cell lines and tumors in which PTEN is lost have elevated levels of activated Akt. Thus, loss of immunohistochemical detection of PTEN might predict for the presence of activated Akt and, in turn, might become useful as a factor predictive of success for therapies directed against this pathway. In general, this type of predictive factor, such as the estrogen receptor, which can predict for the efficacy of a given therapy, such as tamoxifen, has great clinical utility because it directly impacts treatment decisions.

Our results support the candidacy of PTEN as a tumor suppressor gene in prostate cancer progression. Indeed, loss of PTEN expression may be an important negative prognostic indicator. We are currently working on the development of well-characterized rabbit polyclonal or murine monoclonal antibodies that would provide unlimited amounts of antibody capable of reacting with formalin-fixed tissue. It is possible that immunohistochemistry may be the optimal method for evaluating the functional status of *PTEN* because it would detect a loss of PTEN induced by a majority of the mechanisms through which gene products are inactivated.

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Phosphorylation of the PTEN Tail Regulates Protein Stability and Function

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The *PTEN* gene is a tumor suppressor localized in the frequently altered chromosomal region 10q23. The tumor suppressor function of the *PTEN* protein (PTEN) has been linked to its ability to dephosphorylate the lipid second-messenger phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate and, by doing so, to antagonize the phosphoinositide 3-kinase pathway. The PTEN protein consists of an amino-terminal phosphatase domain, a lipid binding C2 domain, and a 50-amino-acid C-terminal domain (the "tail") of unknown function. A number of studies have shown that the tail is dispensable for both phosphatase activity and blocking cell growth. Here, we show that the PTEN tail is necessary for maintaining protein stability and that it also acts to inhibit PTEN function. Thus, removing the tail results in a loss of stability but does not result in a loss of function because the resultant protein is more active. Furthermore, tail-dependent regulation of stability and activity is linked to the phosphorylation of three residues (S380, T382, and T383) within the tail. Therefore, the tail is likely to mediate the regulation of PTEN function through phosphorylation.

The *PTEN* gene was cloned as a candidate tumor suppressor gene from the chromosome 10q23 region, a locus frequently targeted for genetic loss in tumors (24, 26, 42). Somatic inactivation of both *PTEN* alleles and loss of heterozygosity have been demonstrated in a number of tumors including glioblastoma, melanoma, and prostate, breast, and endometrial carcinomas (reviewed in reference 46). Germ line *PTEN* mutations are associated with the development of the related dominantly inherited disorders known as Cowden disease and Bannayan-Zonana syndrome (28–30, 34). These disorders are characterized by the presence of benign hamartomas of the skin, intestinal tract, and central nervous system and by an increased incidence of cancers of the thyroid and breast (28, 29, 34). Similarly, heterozygous *PTEN* mice develop a variety of tumors and proliferative lesions of multiple tissues (10, 11, 37, 43).

Reconstitution of PTEN expression to certain PTEN null cells results in an increase in the population of cells in the G_1 phase of the cell cycle (13, 23, 39); in other PTEN null cells it results in the induction of apoptosis or anoikis (9, 25, 32). Accumulating evidence suggests that these functions are linked to the lipid phosphatase activity of PTEN, which allows PTEN to antagonize the phosphatidylinositol 3-kinase (PI3K) pathway (reviewed in references 4 and 46). A number of downstream targets of phosphatidylinositol 3,4,5-triphosphate and phosphatidylinositol 3,4-bisphosphate including the serine-threonine kinase Akt, BTK, SGK, and $p70^{S6K}$, have been identified (6, 12, 18–20, 27). Akt, in particular, appears to play a role in both proliferative and apoptotic signals. Constitutive activation of Akt has been found in cells that lack functional PTEN, and PTEN can inhibit Akt kinase activity in cells. A number of downstream targets of Akt have been described and include GSK3, BAD, caspase-9, IKKα, and the forkhead transcription factors FKHR, FKHRL1, and AFX (2, 3, 5, 7, 8, 21, 36, 44). Our group has recently found that forkhead transcription factors are inactive in PTEN null cells and that reconstitution of FKHR activity, in the absence of PTEN, can induce both cell cycle arrest and apoptosis in susceptible PTEN null cells (N. Nakamura, S. Ramaswamy, F. Vazquez, and W. Sellers, submitted for publication).

Each molecular constituent of the PI3K pathway, such as receptor tyrosine kinases, PI3K, and Akt, is subjected to regulation of its activity. Likewise, it has been speculated that PTEN might be regulated, but to date evidence of such regulation has remained elusive (4). In keeping with the idea that PTEN might be regulated, protein phosphatases in general are regulated by a number of mechanisms including phosphorylation, second messengers, regulatory subunits, subcellular localization, dimerization, and binding to inhibitory proteins (reviewed in references 1 and 17).

The PTEN protein contains the signature motif (HCXX GXXR) of the family of protein tyrosine phosphatases and dual-specificity phosphatases. The PTEN crystal structure shows that PTEN consists of an amino-terminal phosphatase domain (PD; residues 7 to 185), which includes the phosphatase signature motif, and a lipid binding C2 domain that extends from residues 186 to 351. C2 domains, named for homology to a domain found in protein kinase C (PKC), have been identified in a number of proteins involved in signal transduction or membrane trafficking such as PKC, $cPLA_2$, phospholipase Cs, and synaptotagmins (reviewed in reference 40). C2 domains can play a role in mediating Ca^{2+}-dependent lipid interactions. However, the C2 domain of PTEN is unlikely to bind Ca^{2+}, and its in vitro binding to lipids is independent of Ca^{2+} (22). The last 50 amino acid residues (354 to 403) (referred to herein as the "tail") were not crystallized, and structural prediction programs fail to identify regions of secondary structure. The function of this domain and its relation-

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ship in three dimensions to the remainder of the protein remain unknown.

The PTEN tail is dispensable for PTEN phosphatase activity and for activity in a number of cellular assays including soft-agar colony suppression assays (14, 22; S. Ramaswamy and W. R. Sellers, unpublished data). Here we show that the tail is necessary for maintaining PTEN stability. However, deletion of the tail also results in an increase in activity as measured by the ability of PTEN to induce a G_1 arrest or to induce the transcriptional activity of FKHR. Thus, deletion of the tail does not result in a loss of PTEN function because, while unstable, the resultant protein is more active. We further demonstrate that the tail is a site for PTEN phosphorylation and that phosphorylation of the tail regulates both PTEN stability and activity.

MATERIALS AND METHODS

Plasmids. pCD19, pSG5L, pSG5L-HA-PTEN, pSG5L-HA-PTEN;1-393, pSG5L-HA-PTEN;1-373, pSG5L-HA-PTEN;1-353, pSG5L-HA-PTEN;1-343, pSG5L-HA-PTEN;1-336; pcDNA3-Flag-FKHR, and pGL3-promoter-FasL were described previously (39, 41, 44, 45; Nakamura et al., submitted).

pSG5L-HA-PTEN;360ΔA, pSG5L-HA-PTEN;S370A, pSG5L-HA-PTEN;A4, pSG5L-HA-PTEN;S380A, pSG5L-HA-PTEN;T382A, pSG5L-HA-PTEN;T383A, pSG5L-HA-PTEN;S385A, pSG5L-HA-PTEN;A3, and pSG5L-HA-PTEN;D3 were generated by site-directed mutagenesis using single-stranded DNA generated from pSG5L-HA-PTEN (Muta-Gene; Bio-Rad). The oligonucleotides used for site-directed mutagenesis are the following: 5′-CACCAGATGTggccGACA ATGAAC-3′ (PTEN;S370A), 5′-GATCATTATAGATATgCTGACgCCgCgGA CgCaGATCCAGAGAATGAAC-3′ (PTEN;A4), 5′-CTGATCATTATCgaTAT gCaGACACaACTGACTCTG-3′ (PTEN; S380A), 5′-GATCATTATcgaTATT CTGACgcaACTGACTCTG-3′ (PTEN;T382A), 5′-GATATTCTGACACCgcg GACTCTGATC-3′ (PTEN;T383A), 5′-GATATTCTGACACtACaGACgcaGA TCCAGAG-3′ (PTEN:S385A), 5′-GATCATTATAGATATgCTGACgCCgCgG ACTCTGATCCAGAG-3′ (PTEN;A3), 5′-GATCATTATcGATATgaTGACga CgaTGACTCTGATCCAG-3′ (PTEN;D3).

Antibodies and immunoblotting. HA-11 (Babco), antihemagglutinin (HA) antibody was used for immunoblotting at 1:1,000; C54 anti-PTEN serum was previously described and was used at 1:1,000 dilution (39).

Cells were washed in phosphate-buffered saline, and cellular proteins were extracted in TNN buffer (150 mM NaCl, 50 mM Tris [pH 7.4], 0.5% NP-40) for 20 min at 4°C. Lysates were cleared by centrifugation, and proteins were separated by gel electrophoresis. Immunoblots were obtained essentially as described previously (39). Briefly, membranes were blocked in Tris-buffered saline–0.05% Triton X-100 (TBS-T)–4% (wt/vol) milk for 1 h at room temperature (RT). Membranes were then incubated with primary antibodies diluted in TBS-T–4% (wt/vol) milk for 1 h at RT. Subsequently, membranes were washed with TBS-T and incubated with horseradish peroxidase secondary antibody (1:20,000; Pierce Chemicals) diluted in TBS-T–4% (wt/vol) milk. Membranes were washed in TBS-T, and bound antibody was detected by enhanced chemiluminescence (Pierce Supersignal).

Cell lines, cell culture, and transfection. 786-0 and ACHN renal carcinoma cells and U2-OS osteosarcoma cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 4,500 mg of glucose/ml, 2 mM L-glutamine, 10% fetal clone (HyClone), and penicillin and streptomycin and were maintained at 37°C in a humidified 10% CO_2 atmosphere. 786-0 cells were transfected using Fugene reagent (Boehringer Mannheim), and U2-OS cells were transfected using calcium phosphate (BBS method), as previously described (39).

Pulse-chase labeling. 786-0 cells were transfected with various pSG5L-HA-PTEN plasmids and split into p60 plates. Forty hours after transfection, cells were washed twice with methionine-free DMEM and then incubated for 45 min in methionine-free DMEM with 10% dialyzed fetal bovine serum (DFBS) (Gibco BRL). Cells were then incubated for 45 min with methionine-free DMEM–10% DFBS containing [^{35}S]methionine (150 μCi/ml) (NEN Life Science Products). The medium was then replaced with complete medium. HA epitope-tagged proteins were isolated by anti-HA immunoprecipitation and resolved on a 7.5% polyacrylamide gel. The labeled protein present at each time point was quantified by phosphorimaging and normalized to the amount of protein present at the zero-time point.

Cell cycle assays. Cell cycle assays were performed essentially as previously described (39). Briefly, 786-0 cells were cotransfected with 4 μg of pCD19 plasmid along with pSG5L vector or the relevant pSG5L-HA-PTEN wild-type or mutant plasmid. Forty hours after transfection the cells were harvested with trypsin, stained with fluorescein isothiocyanate-conjugated anti-CD19 antibody (CalTag), fixed in 70% ethanol, and stained with propidium iodide in the presence of RNase A. The cell cycle profile of the CD19-positive cells was determined by two-color fluorescence-activated cell sorting (FACS). Data are shown as the percentages of increase in the G_1 population. This was determined by dividing the absolute percentage difference between the vector control and the experimental data point by the percentage of G_1 cells in the vector and then multiplying by 100.

Luciferase reporter assays. FKHR transactivation assays were performed essentially as described previously (Nakamura et al., submitted). Briefly, cells were transfected in 12-well plates with 0.25 μg of the FasL luciferase and β-galactosidase reporter plasmids, 0.5 μg of pCDNA3-FHKR, and various amounts of pSG5L-HA-PTEN. Cells were lysed 40 h after transfection using 1× reporter lysis buffer by following the manufacturer's instructions (Promega). Luciferase and β-galactosidase activities were measured as described previously (41). Luciferase activity was normalized to β-galactosidase activity. Fold activation was calculated by dividing the normalized luciferase activity by the normalized activity obtained in the presence of the vector and reporter plasmid alone.

Metabolic labeling, proteolytic digestions, and phosphoamino acid analysis. ACHN or transfected U2-OS cells were washed twice with phosphate-free DMEM. Then the medium was changed to a mixture of phosphate-free DMEM, 10% DFBS, and 1 mCi (endogenous) or 200 μCi (transfected) of [^{32}P]orthophosphate (NEN Life Science Products)/ml, and the cells were incubated from 2 to 4 h. Labeled proteins were isolated by immunoprecipitation using HA-11 (transfected) or C54 (endogenous) antibodies, resolved by sodium dodecyl sulfate–7.5% polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane. The phosphorylated proteins were visualized by autoradiography.

Proteolytic digestions were done essentially as described previously (38). Membrane pieces containing phosphoproteins were excised, washed with double-distilled water (ddH_20), and blocked with 0.5% polyvinylpyrrolidone MW360 (PVP-360) in 100 mM acetic acid for 30 min at 37°C. Digestion was performed with 5 μg of sequencing-grade trypsin (Promega) overnight at 37°C. Peptides were twice lyophilized to dryness and washed with ddH_20. Peptides were then resuspended in a small volume of Laemmli sample buffer and then resolved in 16.5% Tris-Tricine gels.

For phosphoamino acid analysis a fraction of the tryptic digested peptide was hydrolyzed in 5.7 N HCl. The resulting amino acids were then lyophilized, washed with ddH_20, and resuspended in a small volume of ddH_20. Samples were then spotted on thin-layer chromatography (TLC) plates (EM Science) together with 1 μg of cold phosphoamino acid standards (Sigma). Phosphoamino acids were then resolved by electrophoresis at pH 1.9 in a buffer containing 2.5% (vol/vol) formic acid (88%) and 7.8% (vol/vol) glacial acetic acid for 45 min at 800 V in the first dimension and by chromatography in the second dimension in a buffer containing 70% (vol/vol) 2-propanol and 15% (vol/vol) HCl. Cold phosphoamino acid standards were visualized by developing the TLC plates with 0.2% (wt/vol) ninhydrin in acetone and baking them at 65°C until color developed.

RESULTS

The PTEN tail modulates PTEN stability. Recently the crystal structure of PTEN has been solved from residues 7 to 353 (eliminating an internal loop of residues 286 to 309). This truncated protein has in vitro lipid phosphatase activity comparable to that of full-length PTEN (PTEN;WT) and can induce apoptosis in LNCaP cells to the same extent as the wild type (14, 22). Similarly, our group mapped the minimal in vivo functional domain of PTEN by C-terminal and N-terminal deletion mutations (S. Ramaswamy and W. R. Sellers, unpublished data). We found that a truncated PTEN protein of residues 10 to 353 retained protein and lipid phosphatase activity in vitro and was able to induce a G_1 arrest in cells. Furthermore, PTEN;1-353 was comparable to PTEN;WT in suppressing soft-agar colony formation in PTEN null renal carcinoma cells (786-0 cells) (S. Ramaswamy and W. R. Sellers, unpublished data). These results indicate that the last 50 amino acids of PTEN are not necessary for lipid or protein phosphatase activity or for its ability to inhibit proliferation or induce apoptosis. For simplicity we refer to these last 50 residues as the PTEN tail (Fig. 1A).

In our experiments we noted that PTEN;1-353 was produced at markedly reduced steady-state levels compared to PTEN;WT. To determine whether the changes in the steady-state protein levels were related to changes in protein stability, 786-0 cells (PTEN null) transiently transfected with plasmids encoding PTEN;WT or PTEN;1-353 were pulse-labeled with [^{35}S]methionine for 45 min. HA-PTEN and HA-PTEN;1-353 were recovered by immunoprecipitation and detected by autoradiography. In these experiments the half-life of PTEN;1-353

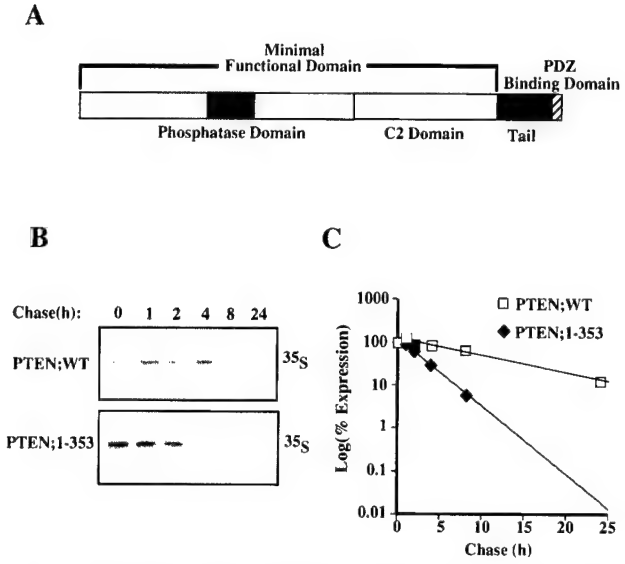

FIG. 1. The PTEN tail is required for protein stability. (A) Schematic representation of the PTEN protein. Dark gray box, phosphatase signature motif (HCXXGXXR); light gray box, PD; white box, C2 domain; black box, C-terminal 50-residues (PTEN tail). The minimal domain that is functional as a growth suppressor is shown. (B) Pulse-chase analysis of PTEN;WT and PTEN;1-353. 786-0 cells were metabolically labeled with [^{35}S]methionine for 45 min. The medium was then replaced with complete growth medium at time zero, and cells were harvested at the indicated times. HA-PTEN and HA-PTEN;1-353 were immunoprecipitated from labeled cell extracts, separated by gel electrophoresis, and detected by autoradiography. (C) Log plot of the percentage of the time-zero protein remaining at the times indicated on the *x* axis. [^{35}S]methionine-labeled, immunoprecipitated protein (B) was quantified by phosphorimager analysis.

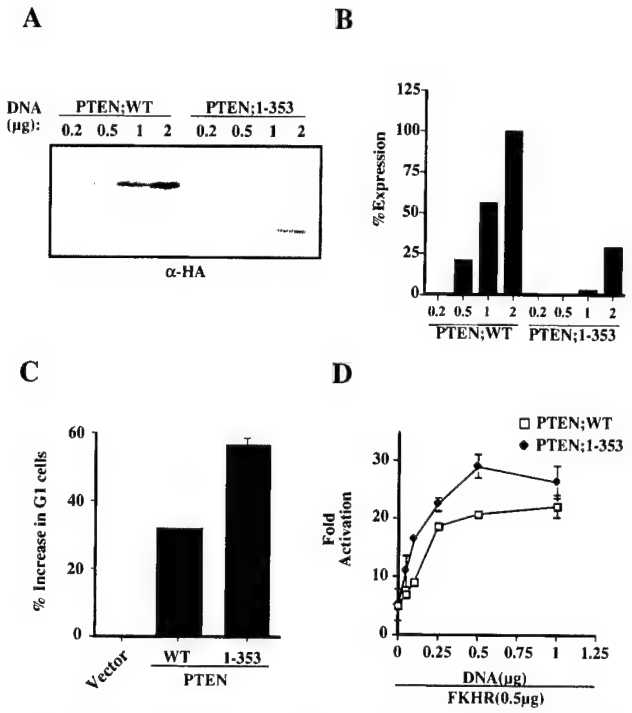

FIG. 2. The PTEN tail is an inhibitory domain. (A) Steady-state protein levels of PTEN;WT and PTEN;1-353. 786-0 cells were transfected with the indicated amounts of plasmids encoding HA-PTEN;WT or HA-PTEN;1-353. Forty hours after transfection, cell lysates were prepared and separated by gel electrophoresis. HA-PTEN and HA-PTEN;1-353 were detected by anti-HA immunoblotting. (B) Quantification of the immunoblot shown in panel A. The radiograph was digitized, and the relative protein quantities were determined using ImageQuant software. The results are expressed as a percentages of PTEN;WT at 2 μg of input plasmid DNA. (C) Induction of G_1 arrest by PTEN;WT and PTEN;1-353. 786-0 cells were cotransfected with a plasmid encoding the cell surface marker CD19 (pCD19) along with plasmids encoding PTEN;WT (0.5 μg) or PTEN;1-353 (2 μg). Forty hours after transfection, the cell cycle distribution of the CD19-positive cells was determined by FACS analysis. Shown are the means and standard errors of duplicate experiments. These data are representative of three independent experiments. (D) Induction of FKHR transcriptional activity by PTEN;WT and PTEN;1-353. 786-0 cells were transfected with a FasL promoter luciferase reporter plasmid and a plasmid encoding FKHR in combination with the indicated amounts of plasmid encoding PTEN; WT or PTEN;1-353. Forty hours after transfection, luciferase activity was determined as described in Materials and Methods. Shown are the means and standard errors of the fold activation relative to the activity obtained with the reporter alone.

was found to be reduced by more than fourfold compared to that of PTEN;WT (Fig. 1B and C). In keeping with these results, it was recently reported that the steady-state level of PTEN;1-351 is reduced compared to that of PTEN;WT when the protein is produced by transfection in COS-7 cells (14). Together these data suggest that the PTEN tail, while not required for the functional activity of the protein, is required for maintaining stability.

The tail domain modulates PTEN biological activity. As a result of the reduced half-life, PTEN;1-353 is produced at significantly lower levels than PTEN;WT. In order to determine whether the resulting decrease in protein production results in reduced activity, PTEN;1-353 and PTEN;WT were compared in a cell cycle arrest assay that reflects the ability of PTEN to act as a lipid phosphatase (39). 786-0 cells were transfected with increasing doses of plasmids encoding PTEN;WT and PTEN;1-353 along with a plasmid encoding the cell surface marker pCD19. The cell cycle distribution of the CD19-positive cells (as a marker of transfection) was determined by staining with fluorescein isothiocyanate-conjugated anti-CD19 and propidium iodide followed by two-color FACS. Surprisingly, at equivalent input plasmid concentrations PTEN;1-353 reproducibly induced a greater increase in G_1 than PTEN;WT (data not shown). Next, the activities of PTEN;WT and PTEN;1-353 were compared when the proteins were produced at similar steady-state levels. Plasmid titration indicated that equivalent protein levels were obtained at 2 μg of PTEN;1-353 and 0.5 μg of PTEN;WT (Fig. 2A and B). At these levels PTEN;1-353 induced a significantly more robust G_1 arrest (Fig. 2C).

Forkhead transcription factors are targets of Akt regulation both in mammalian cells and in *Caenorhabditis elegans* (2, 3, 15, 21, 33, 35). Akt phosphorylation creates 14-3-3 binding sites and results in the cytoplasmic localization of forkhead proteins. Furthermore, recent data in our laboratory have shown that in PTEN null cells FKHRL1 and exogenously expressed FKHR are retained in the cytoplasm and that exogenously expressed FKHR fails to activate transcription. Coexpression of wild-type PTEN, but not a PTEN mutant lacking lipid phosphatase activity, with FKHR restores cytoplasmic localization and FKHR transactivation as measured with a 3XIRS promoter or FasL promoter luciferase reporters (Nakamura et al., submitted). Thus, FKHR transcriptional activity requires PTEN function. The induction of FKHR transcriptional activity by PTEN is also dose dependent, as shown in Fig. 2D.

We next compared PTEN;WT and PTEN;1-353 in a FKHR transcriptional activation assay. Consistent with the results obtained in the cell cycle assay (Fig. 2C), the ability of PTEN;1-353 to induce FKHR transcriptional activity was enhanced compared to that of PTEN;WT. Furthermore, at every DNA

plasmid concentration tested, PTEN;1-353 induced FKHR activation more efficiently than PTEN;WT, although protein levels were reduced by more than fourfold. These results suggest that the PTEN tail not only plays a role in maintaining its protein stability but also in regulating its biological activity. Specifically, these data suggest that the tail acts to restrict or inhibit PTEN function.

PTEN is a phosphoprotein. The PTEN tail is rich in serine and threonine (28% of the residues) and contains consensus phosphorylation sites for GSK3, PKA, CK1, and CK2. In order to determine whether regulation of PTEN stability and activity might be linked to phosphorylation, we determined whether endogenous PTEN is phosphorylated. To this end, ACHN renal carcinoma cells that contained PTEN were metabolically labeled with [^{32}P]orthophosphate. Labeled lysates were incubated with an anti-PTEN antibody (C54) or a preimmune control. Bound proteins were separated by gel electrophoresis and detected by autoradiography. A ^{32}P-labeled protein of the same molecular weight as PTEN was detected in anti-PTEN immunoprecipitates but not in the preimmune control (Fig. 3A). In separate experiments in which the labeled proteins were transferred to nitrocellulose, this ^{32}P-labeled species comigrated with PTEN, as detected by immunoblotting. These data suggest that endogenous PTEN is phosphorylated. Next, PTEN plus U2-OS cells were transfected with either the empty vector (pSG5L) or pSG5L-HA-PTEN and metabolically labeled with orthophosphate. Labeled cells were lysed, and epitope-tagged proteins were immunoprecipitated with anti-HA antibody. In parallel, endogenous PTEN was immunoprecipitated from lysates prepared from untransfected orthophosphate-labeled U2-OS cells. A phosphorylated protein of 58 kDa was detected in the anti-HA immunoprecipitates from cells expressing HA-PTEN but not in the vector-transfected cells (Fig. 3B). Next, phosphorylated endogenous PTEN and exogenously produced HA-PTEN were digested with trypsin. As shown in Fig. 3C, an identical pattern of phosphotryptic peptides was observed when phosphopeptides were separated by Tris-Tricine gel electrophoresis (16.5% acrylamide). Phosphoamino acid analysis of both endogenous and transfected PTEN proteins showed phosphorylation of serine and threonine residues, while tyrosine phosphorylation was not detected (Fig. 3D). These data suggest that endogenous PTEN is a phosphoprotein, that HA-PTEN produced by transfection is a phosphoprotein, and that these proteins are phosphorylated on the same peptides, predominantly on serine and threonine.

PTEN is phosphorylated within the tail domain. To determine the exact sites of phosphorylation, U2-OS cells were transfected with plasmids encoding a series of PTEN C-terminal deletion mutants and labeled with orthophosphate. ^{32}P-labeled HA-tagged proteins were recovered by anti-HA immunoprecipitation and detected by autoradiography. These experiments revealed that deletion of residues 354 to 403 (the tail) abrogated most PTEN phosphorylation (Fig. 4A). In addition, gel electrophoretic separation of peptides generated by cyanogen bromide cleavage of orthophosphate-labeled HA-PTEN revealed a single phosphorylated peptide consistent in size with the predicted CNBr peptide containing the PTEN tail (data not shown). Next, every serine and threonine in the tail was mutated either singly or in clusters to alanine. These PTEN mutants were transfected into U2-OS cells and labeled with orthophosphate. No single-amino-acid substitution abrogated or significantly reduced the total phosphorylation of PTEN (data not shown). However, the substitution of a serine/threonine cluster, S380, T382, T383, and S385 (the A4 mutant), did significantly alter total PTEN phosphorylation. In addition, mutation of these sites led to the loss of the more

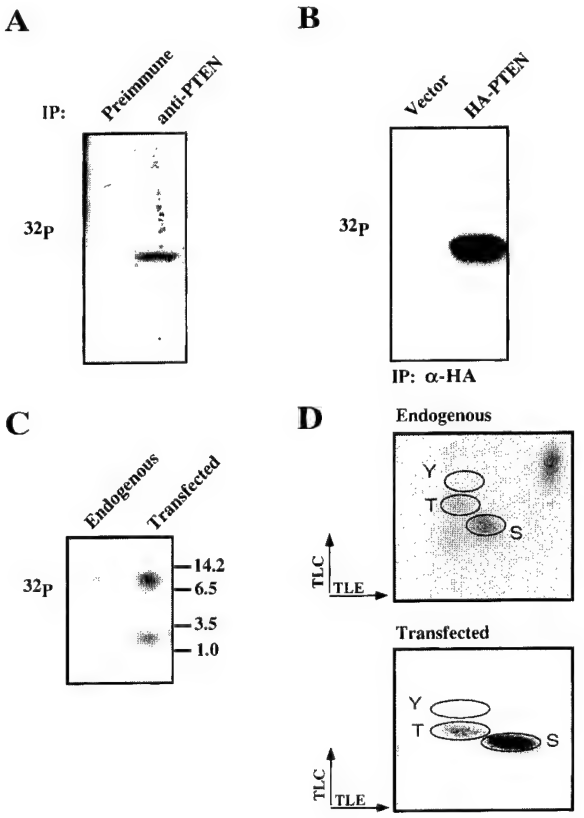

FIG. 3. PTEN is a phosphoprotein. (A) Phosphorylation of endogenous PTEN. Asynchronously growing ACHN cells were metabolically labeled with [^{32}P]orthophosphate for 4 h. Protein extracts were prepared and immunoprecipitated with either preimmune or anti-PTEN (C54) antibody. Bound proteins were resolved by gel electrophoresis, transferred to a nitrocellulose membrane, and detected by autoradiography. Data shown are from the same exposure of the same gel; the lanes were rearranged for clarity. (B) Phosphorylation of exogenous HA-PTEN. U2-OS cells were transfected with the backbone vector or pSG5L-HA-PTEN. Forty hours after transfection cells were labeled with ^{32}P-orthophosphate for 2 h. Protein extracts were prepared and immunoprecipitated (IP) with anti-HA antibody. Bound proteins were resolved and detected as for panel A. (C) Tryptic phosphopeptides of endogenous PTEN and exogenously produced HA-PTEN. The bands corresponding to endogenous PTEN or HA-PTEN (A and B) were excised, digested with trypsin, and resolved on a 16.5% Tris-Tricine gel. Phosphopeptides were detected by autoradiography. (D) Phosphoamino acid analysis of endogenous PTEN and exogenously produced HA-PTEN. Tryptic digest products of in vivo-labeled endogenous PTEN or HA-PTEN were hydrolyzed with acid, and the resulting phosphoamino acids were resolved by two-dimensional thin-layer electrophoresis and detected by autoradiography. Phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) standards were visualized by ninhydrin staining.

slowly migrating tryptic phosphopeptide (Fig. 4B). This peptide therefore is likely to be peptide 2 (Fig. 4D). In contrast, substitution of alanines for the serine/threonine cluster beginning at S360 (which contains a GSK3 consensus phosphorylation site) had no effect on either the total phosphate incorporated into PTEN or the phosphorylation of the two phosphopeptides detected in Tris-Tricine gels (Fig. 4B). Next, the A4 mutation was combined with a single alanine substitution at a consensus CK2 site (S370) found in peptide 1 of the tail to give the A5 mutation (Fig. 4D). When PTEN;A5 was expressed in U2-OS cells and tested for phosphorylation, ^{32}P labeled protein was not detected despite adequate protein expression (Fig. 4C). Phosphopeptide analysis was not possible because no labeled protein could be excised from the gel. These results indicate that most, if not all, of the PTEN tail

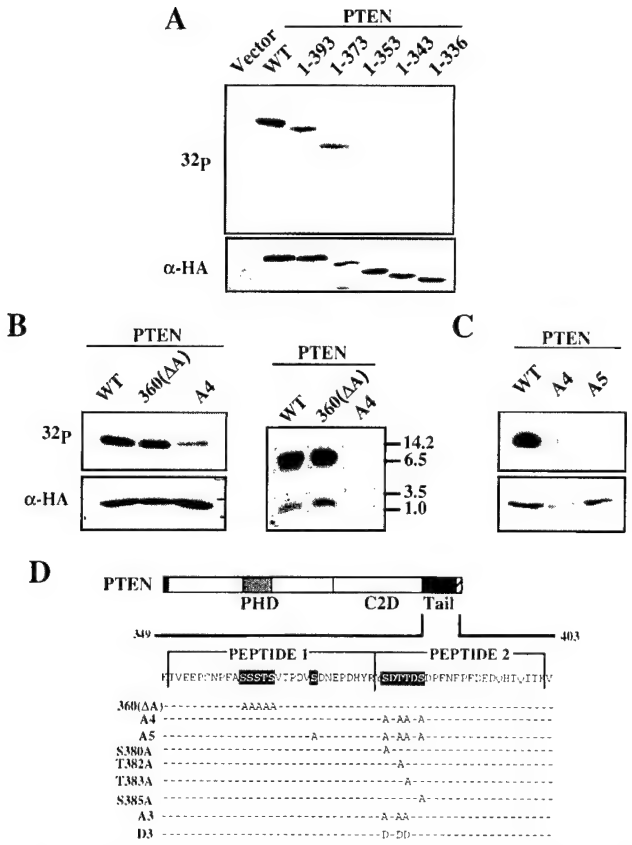

FIG. 4. PTEN is phosphorylated within the tail. (A) Deletion of the tail impairs PTEN phosphorylation. Plasmids encoding either wild-type (WT) HA-PTEN or the indicated C-terminal truncation mutants were transfected into U2-OS cells. Twenty-four hours after transfection, cells were split into T25 flasks and 35-mm plates. Forty hours after transfection, T25 flasks were metabolically labeled with [^{32}P]orthophosphate; anti-HA immunoprecipitates of protein extracts were prepared, and bound labeled proteins were detected by autoradiography (top). In parallel, whole-cell extracts prepared from the 35-mm plates were separated by gel electrophoresis, transferred to nitrocellulose, and immunoblotted with anti-HA antibody (bottom). (B [left] and C) Alanine mutations in the PTEN tail impair phosphorylation. U2-OS cells were transfected with plasmids encoding PTEN;WT or the indicated alanine substitution mutants. Transfected cells were split, replated, grown overnight, and used in parallel for metabolic labeling and for the preparation of whole-cell extracts. [^{32}P]orthophosphate labeling, immunoprecipitation, and autoradiography (top) were performed as for panel A. Anti-HA immunoblotting (bottom) was performed as for panel A. (B [right]) Tryptic phosphopeptides of PTEN tail substitution mutants. Phosphopeptides resulting from the tryptic digestion of either HA-PTEN;WT or the indicated mutant proteins were resolved on Tris-Tricine gels and detected as for Fig. 3C. (D) Schematic representation of PTEN tail substitution mutants used. Dashes indicate amino acids that were not altered. The predicted tryptic peptides of the PTEN tail are shown as peptide 1 and peptide 2. PHD, phosphatase domain; C2D, C2 domain.

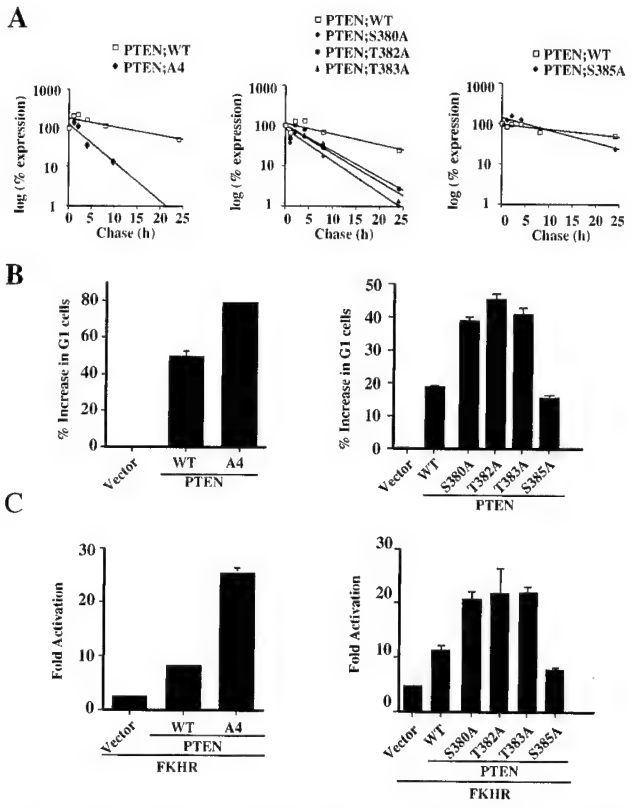

FIG. 5. Mutation of phosphoacceptor sites in the PTEN tail alters protein stability and activity. (A) Protein half-life of PTEN;A4 (left) and single-substitution mutants (middle and right). 786-0 cells were transfected with plasmids encoding HA-PTEN;WT and HA-PTEN;A4 or the indicated single-substitution mutants, metabolically labeled with [^{35}S]methionine, and chased for the indicated times. Data are shown as in Fig. 1C. (B) Increased activity of PTEN;A4 (left) and mutants with at single substitution of S380, T382, or T383 (right) in the cell cycle arrest assay. 786-0 cells were cotransfected with a plasmid encoding CD19 (pCD19) along with plasmids encoding PTEN;WT, PTEN;A4, or single-substitution mutants at concentrations resulting in equivalent protein production (0.5 μg of pSG5L-HA-PTEN;WT and pSG5L-HA-PTEN;S385A and 2.0 μg of pSG5L-HA-PTEN; A4, pSG5L-HA-PTEN;S380A, pSG5L-HA-PTEN;T382A, and pSG5L-HA-PTEN;T383A). Forty hours after transfection the cell cycle distribution of the CD19-positive cells was analyzed as for Fig. 2C. These data are representative of three independent experiments. (C) FKHR transcriptional activity induced by PTEN;A4 (left) and single-substitution mutants (right). 786-0 cells were transfected with a plasmid encoding FKHR along with either the backbone vector or plasmids encoding PTEN;WT, PTEN;A4, or single-substitution mutants as indicated. Forty hours after transfection luciferase activity was measured and the fold activation of FKHR relative to the activity obtained with the reporter alone was calculated as for Fig. 2D. Shown are the means and standard errors of experimental duplicates. These data are representative of two independent experiments.

phosphorylation occurs on serine 370 and one or more sites of the A4 cluster (S380, T382, T383, and S385).

To directly identify the PTEN phosphorylation sites, 2 μg of HA-PTEN isolated by anti-HA immunoaffinity purification was digested with trypsin and analyzed by LC/MSMS. Here, peptide 1 with a phosphoserine at residue 370 was identified. However, peptide 2 was not detectable in either a phosphorylated or unphosphorylated state. No other phosphopeptides were identified (data not shown).

Mutation of the phosphorylation sites in the tail alter PTEN stability and biological activity. To determine whether phosphorylation of one or more of the amino acid residues delineated above has a role in modulating PTEN stability, the relevant phosphorylation site mutants were produced in U2-OS cells by transient transfection and the steady-state levels of HA-PTEN and the phosphorylation mutants were determined by immunoblot analysis. While mutation of serine 370 did not change the steady-state level of PTEN (data not shown), mutation of the S/T cluster (PTEN;A4) resulted in a marked decrease in the steady-state protein level (data not shown). Furthermore, pulse-chase labeling experiments revealed a marked reduction in the PTEN;A4 half-life (Fig. 5A, left).

As deletion of the tail led to an increase in PTEN activity, we next asked if the PTEN;A4 mutant was similarly more active in biological assays. In keeping with the data for the PTEN tail, the PTEN;A4 mutant, while expressed at lower levels, was more active in both inducing a G_1 arrest in PTEN null 786-0

cells (Fig. 5B, left) and inducing FKHR transcriptional activation (Fig. 5C, left). These data suggest that phosphorylation within the A4 cluster is required to maintain stability and is linked to an inhibitory activity of the PTEN tail.

Next, the individual point mutations within the A4 cluster were tested in the same assays of protein half-life and activity. While replacement of serine 385 with alanine did not alter the steady-state PTEN protein levels, mutation of S380, T382, and T383 each reduced both the steady-state protein levels and the protein half-life (Fig. 5A, right, and data not shown). More specifically, the half-life of the PTEN;A4 mutant was reduced more than six-fold compared to that of PTEN;WT. Similarly, mutating serine 380 reduced the half-life by more than fivefold. Mutation of threonines 382 and 383 reduced PTEN half-life by 2.7- to 3-fold. Furthermore, the individual phosphorylation mutants with substitutions S380A, T382A, and T383A, but not S385A, were again more active in inducing a G_1 arrest and in inducing FKHR transcriptional activation (Fig. 5B and C, right). Taken together, these data show that the increased activity associated with deletion of the tail is entirely mimicked by mutations within the A4 cluster, specifically S380, T382, or T383.

The above data raised the possibility that phosphorylation of these three specific residues (S380, T382, and T383) might be required to maintain PTEN in a stable yet relatively inactive state. While mutation of each of these individual residues did not alter total incorporation of ^{32}P into the PTEN protein (data not shown) when the S380, T382, and T383 residues were mutated to alanine (PTEN;A3) incorporation of ^{32}P into PTEN during orthophosphate labeling was reduced (Fig. 6A) and the most slowly migrating tryptic phosphopeptide (peptide 2) was not detectable (Fig. 6B). In keeping with the data for PTEN;A4 and for the individual phosphorylation site mutants (with mutations S380A, T382A, and T383A), PTEN;A3 was found to have a reduced protein half-life, to be expressed at lower steady-state levels, and to be more active than wild-type PTEN in biological assays (Fig. 6C to F).

Aspartic acid substitutions at the phosphorylation sites in the PTEN tail lead to a recovery of PTEN stability. A reasonable interpretation of these results is that the serine/threonine-to-alanine substitutions of PTEN block phosphorylation and thereby alter the stability and activity of PTEN in cells. On the other hand, it is formally possible that mutation of these residues might result in these changes independent of the changes in PTEN tail phosphorylation. To distinguish these possibilities, conversion of the serines/threonines to aspartic acid was used to try and mimic phosphorylation of these residues. As we had noted that mutation of any one of the putative phosphorylation sites altered both stability and activity, it appeared that phosphorylation of all three residues might be required for maintaining PTEN stability. Therefore, we generated a PTEN;D3 cDNA in which codons 380, 382, and 383 encoded aspartic acid.

In contrast to the results obtained with PTEN;A3, PTEN;D3 recovered the expression levels of PTEN;WT, suggesting that a negative charge was enough to maintain protein stability (Fig. 6C). To test this possibility, we performed a pulse-chase experiment to determine if aspartic acid substitution could restore PTEN stability. As shown in Fig. 6D, PTEN;D3 recovered the stability of PTEN;A3 and was similar to PTEN;WT. As expected, when analyzed after orthophosphate labeling, PTEN;D3 like PTEN;A3 was found not to contain phosphopeptide 1 (data not shown). These data argue that the D3 mutation does not restore stability simply be restoring phosphorylation of PTEN at other sites but rather that phosphorylation of the S380 cluster is required for appropriate stability.

Next, we asked whether aspartic acid substitution led to a change in the activity of PTEN in the cell cycle assay and the FKHR transactivation assay. As would be predicted if loss of phosphorylation was responsible for the changes seen in PTEN activity, PTEN;D3 recovered the activity of PTEN;WT and its activity was reduced compared to that of PTEN;A3 (Fig. 6E-F).

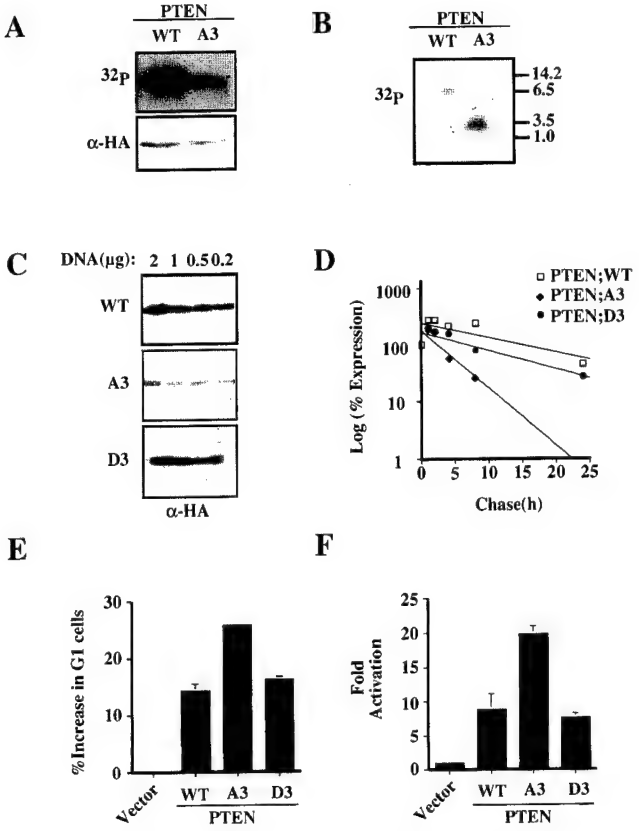

FIG. 6. Aspartic acid substitutions of serine 380, threonine 382, and threonine 383 restore PTEN expression levels and half-life. (A) Substitution mutations of serine 380, threonine 382, and threonine 383 impair PTEN phosphorylation. Plasmids encoding PTEN;WT and PTEN;A3 were transfected into U2-OS cells, and phosphorylation of the resulting HA-PTEN proteins was determined as for Fig. 4A. (B) Phosphorylated tryptic peptides of the PTEN tail substitution mutants. Phosphopeptides resulting from the tryptic digestion of HA-PTEN;WT or HA-PTEN;A3 were resolved on Tris-Tricine gels and detected by autoradiography. (C) Aspartic acid substitution restores HA-PTEN steady-state protein levels. 786-0 cells were transfected with the indicated amounts of plasmids encoding HA-PTEN;WT and HA-PTEN;A3 and HA-PTEN;D3 mutants. Forty hours after transfection whole-cell extracts were analyzed by immunoblotting with anti-HA antibody. (D) Aspartic acid substitution restores PTEN stability. 786-0 cells were transfected with plasmids encoding HA-PTEN;WT, HA-PTEN;A3, or HA-PTEN;D3, metabolically labeled with [^{35}S]methionine, and chased for the indicated time. Data are shown as in Fig. 1C. (E) Cell cycle arrest induced by PTEN;WT or the indicated substitution mutants. 786-0 cells were transfected with plasmids encoding PTEN;WT or the indicated PTEN mutants. Forty hours after transfection the cell cycle distribution of the CD19-positive 786-0 cells was determined as for Fig. 2C. Data are the means and standard errors of experimental duplicates. The data are representative of three independent experiments. (F) FKHR transcriptional activation. 786-0 cells were transfected with the FasL promoter luciferase reporter plasmid and a plasmid encoding FKHR alone or in combination with a plasmid encoding PTEN;WT or the indicated PTEN substitution mutants. Forty hours after transfection the fold activation of reporter activity was determined as for Fig. 2D. The data are the means and standard errors of experimental duplicates. These data are representative of two independent experiments.

DISCUSSION

Recently it was shown that PTEN;1-351 has in vitro lipid phosphatase activity, can inhibit Akt activation, and can sup-

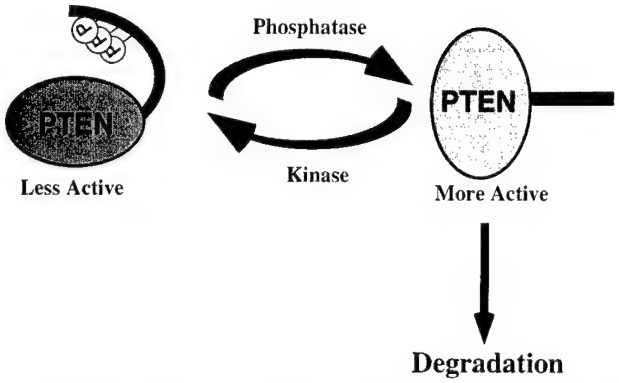

FIG. 7. Model for PTEN regulation by phosphorylation of the tail. The black extension to PTEN represents the tail, and the circles labeled P represent phosphorylated residues within the tail. PTEN phosphorylation on the tail would restrict PTEN activity. Dephosphorylation of the tail would result in an increase in PTEN activity and in its rapid degradation.

press anchorage-independent growth (14). In accordance with these results, we found that PTEN;1-353 inhibits the growth of PTEN null 786-0 cells in soft agar and induces apoptosis in LNCaP cells (S. Ramaswamy and W. R. Sellers, unpublished data). Furthermore, the PTEN crystal structure shows that these residues include the entire PD and a C2 lipid binding domain (22). Together these data suggest that the tail (residues 354 to 403) is not required for biological activity of the protein.

Here, we have shown that deletion of the PTEN tail results in a loss of protein stability. One might expect that loss of stability would lead to a loss of PTEN function; however, as stated above, no requirement for the tail was found in multiple assays of PTEN function. This paradox can be explained by the concomitant loss of an inhibitory activity that maps to the tail region. Thus, the tail contains sequences that are required for protein stability and for negative regulation of PTEN function. This linkage between stability and activity argues for a model in which PTEN is normally found in a stable yet relatively inactive state. In such a model, activation of PTEN would be accompanied by a decrease in protein half-life, presumably reflecting degradation of the active molecule (Fig. 7). Such a mechanism would prevent the untimely or promiscuous activation of PTEN. The linkage between protein activation and protein instability is a common theme in molecular biology. Examples of proteins where the activated form of the protein is unstable include Src, EGFR, PDGFR, and the nuclear receptors RAR and RXR (16, 31, 47).

The tail is also required for PTEN phosphorylation. In vitro mutagenesis revealed specific phosphorylation of S380, T382, and T383. Mutation of these sites led to a loss of stability and a gain in PTEN function. Conversely, aspartic acid mutations of these same residues preserved the protein half-life and the function of PTEN. Together, these data argue strongly that phosphorylation of these residues is required for stability and that the changes in stability are not simply the result of a misfolding secondary to changes in the amino acid residues. Recently others have shown that mutations in the C2 domain can reduce expression levels and protein half-life (14). The mutants are, however, found to be functionally inactive or significantly impaired in biological assays; thus the instability of these mutants might indeed arise as a consequence of protein misfolding (14, 22). On the other hand, the PTEN;A3 mutant, while unstable, is more active, arguing strongly against protein misfolding as a mechanism for instability in this instance.

What is the mechanism by which PTEN phosphorylation regulates protein stability? As pointed out by Georgescu et al. (14) the tail contains two putative PEST sequences (residues 350 to 375 and 379 to 386) implicated in targeting proteins for proteolytic degradation. We have found that the second PEST region is the site of three phosphorylation sites (S380, T382, T383), raising the possibility that this sequence is normally masked by phosphorylation. Arguing against this model, however, is the fact that deletion of the entire tail also results in a shorter half-life. Secondary structure prediction and the results of proteolytic digestion experiments suggest that the tail is a relatively unstructured and presumably flexible region. Thus, one model is that the tail can mask a degradation signal present elsewhere in the PTEN protein when phosphorylated; this signal would be unmasked by dephosphorylation leading to a shift in the position of the tail (Fig. 7).

As an alternative model, the tail might regulate PTEN localization through interactions with the adjacent C2 domain. If so, then stability might simply reflect the localization of PTEN to a subcellular compartment where PTEN degradation can take place. To date we have not seen an obvious effect on the membrane localization of the relevant C-terminal truncations or phosphorylation site mutants (data not shown). Surprisingly, PTEN;1-353 and PTEN;A4 manifestly increased localization to the nucleus (data not shown). Whether this apparent change in localization results from a more rapid degradation of the cytoplasmic component of these mutants or from a true shift in localization is not yet clear. Nonetheless, regulation of localization by tail phosphorylation might play an important role in the regulation of PTEN.

What is the mechanism through which PTEN function is regulated by the tail? There are a number of mechanisms that could account for the inhibitory activity of the tail on PTEN. First, the phosphatase activity itself could be regulated through an allosteric or steric mechanism. To date, however, we have seen no effects of phosphorylation site mutations on intrinsic phosphatase activity (data not shown). Second, the ability of PTEN to gain access to a substrate could be altered either through changes in localization (see above), through changes in the position of the tail, or perhaps through inhibitory interactions with tail-associated proteins. With respect to the last idea, the PTEN tail contains a PDZ binding domain. Given that the PDZ binding sequence (along with the entire tail) is dispensable for the biological function of PTEN, it would seem likely that this domain is linked to the role for the tail that we have put forth. Specifically, the interaction of a PTEN with a PDZ domain-containing protein might be regulated through PTEN phosphorylation.

Are the PTEN phosphorylation events that we have identified constitutive or regulated? Our data are most consistent with the idea that PTEN exists in a predominantly phosphorylated state and that dephosphorylation of the S380 cluster is a regulated event. While PTEN runs as a single band under standard sodium dodecyl sulfate-gel electrophoresis conditions, on two-dimensional gels multiple isoforms of PTEN can be distinguished based on differences in the isoelectric point (data not shown). While it is likely that these forms represent different PTEN phosphoisoforms, how these forms are related to each phosphorylation site or to PTEN activity or stability is not yet known. Our model suggests that PTEN function is regulated by the balance between a kinase and a phosphatase. It is possible that the kinase constitutively phosphorylates PTEN and that a phosphatase regulates the activity. An intriguing possibility is that PTEN activates itself through autodephosphorylation maintaining a constant loop of activity. Identification of the kinase and phosphatase activities respon-

sible for the regulation of these sites should provide further insights into how regulation of PTEN is achieved.

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Forkhead transcription factors are critical effectors of cell death and cell cycle arrest downstream of PTEN

running title: FKHR is a critical downstream effector of PTEN function

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Abstract

PTEN acts as a tumor suppressor, at least in part, by antagonizing phosphoinositide 3-kinase (PI3K) /Akt signaling. Here, we show that forkhead transcription factors FKHRL1 and FKHR, substrates of the Akt kinase, are aberrantly localized to the cytoplasm and can not activate transcription in PTEN-deficient cells. Restoration of PTEN function restores FKHR to the nucleus and restores transcriptional activation. Expression of a constitutively active form of FKHR that can not be phosphorylated by Akt produces the same effect as reconstitution of PTEN to PTEN deficient tumor cells. Specifically, activated FKHR induces apoptosis in cells that undergo PTEN-mediated cell death and induces G1 arrest in cells that undergo PTEN-mediated cell cycle arrest. Furthermore, both PTEN and constitutively active FKHR induce $p27^{KIP1}$ protein, but not p21. These data suggest that forkhead transcription factors are a critical effectors of PTEN-mediated tumor suppression.

Introduction

The *PTEN/MMAC/TEP-1* tumor suppressor gene (hereafter referred to as *PTEN*) is a common target of somatic mutation in a number of malignancies including prostate and endometrial cancer, glioblastoma and melanoma (7, 27, 35, 37, 39, 55, 64, 68, 70, 74). In addition, germ line mutations in the *PTEN* gene are associated with the development of Cowden disease, an inherited hamartoma syndrome associated with an elevated risk of breast and thyroid cancer (38, 46). The *PTEN* protein product (PTEN) functions as both a protein and lipid phosphatase (40, 45). The former activity is associated with inhibition of cell spreading and dephosphorylation of FAK (66). PTEN lipid phosphatase activity is specific for the 3 position of phosphatidylinositol-3,4,5-trisphosphate and phosphatidylinositol-3,4-bisphosphate; both of which are by-products of the lipid kinase activity of the phosphoinositide-3 kinase (PI3K) (40). This latter PTEN activity is associated with the ability of PTEN to antagonize signaling through the PI3K pathway and hence block inappropriate activation of the serine threonine kinase Akt (reviewed in references (8) and (72)).

Reintroduction of PTEN into certain PTEN null tumor cells, such as U87-MG and 786-O, leads to the induction of a G1 arrest (22, 34, 54). This arrest requires the lipid phosphatase activity of PTEN and can be overridden by a constitutively active form of the Akt, a downstream effector of PI3K (22, 54). In keeping with these data, *PTEN* heterozygosity results in excessive proliferation in murine prostate and thyroid tissues; *PTEN-/-* embryos have widespread excess BrdU incorporation, and *PTEN-/-* ES cells show abnormal cell-cycle kinetics and reduced

$p27^{KIP1}$(p27) levels (20, 63, 65). These data demonstrate a necessary role for PTEN in cell-cycle regulation.

Introduction of PTEN into certain other PTEN null tumor cells such as LNCaP, MDA-MB-468 and U251 results in the induction of apoptosis or anoikis (15, 16, 36, 44). This induction is also tied to inhibition of PI3K and Akt (36, 44). Further, the study of murine *PTEN* loss-of-function alleles has revealed defects in apoptosis. *PTEN*-/- murine fibroblasts are impaired in their response to apoptotic stimuli such as UV irradiation and osmotic stress (63). *PTEN*+/- mice have abnormal lymphoid aggregates and lymphocytes from these mice have reduced Annexin V staining, a marker of apoptosis (53). Finally, PTEN+/- mice also develop a lymphoproliferative syndrome that results from, and phenocopies, defects in Fas signaling (19). Collectively, these data support a necessary role for PTEN in mediating apoptosis in fibroblasts and lymphocytes. PTEN, like p53, is therefore a regulator of both cell-cycle progression and apoptosis.

Potential effectors of PI3K signaling, downstream of PTEN, include a number of identified Akt substrates such as BAD, Caspase 9, IKKα and the Ffd transcription factors FKHR, FKHRL1, and AFX (5, 6, 9, 14, 32, 49, 67). Each of these substrates is implicated in cell-survival. Other downstream targets of Akt include nitric oxide synthetase, GSK-3 and 4E-BP1/Phas-I (13, 25, 42). While each of these proteins is a known Akt substrate, with respect to the function of PTEN as a tumor suppressor it is not known which substrates are either necessary and/or sufficient for enacting cell-cycle control or for the induction of apoptosis. One possibility

is that different Akt substrates are responsible for enacting cell cycle control and regulating apoptosis. Alternatively, it is possible that one Akt target might be critical for both functions. Therefore, we sought to determine whether one or more of these substrates was deregulated in PTEN null tumors, and in addition, to determine whether any one target was either necessary or sufficient for PTEN to regulate the cell cycle or to induce apoptosis.

Here, we show for the first time that members of the forkhead transcription factor family are deregulated and inactive in PTEN null cells. Furthermore, a form of the Forkhead factor FKHR (FKHR;AAA) that can not be phosphorylated by Akt is sufficient to induce apoptosis in PTEN null cells. In addition, this constitutively active form of FKHR induced a cell-cycle arrest rather than apoptosis in PTEN null cells that likewise undergo a G1 arrest following restoration of PTEN function. As shown before for PTEN, the phosphosite mutant form of FKHR was also capable of inducing p27, but not p21 protein. These data suggest that an active form of FKHR can compliment PTEN deficiency in both cell cycle and apoptotic pathways and suggest that FKHR may function as regulator of both proliferation and cell survival in the PI3K signaling pathway. These results further suggest that FKHR or its related family members AFX and FKHRL1 are critical proteins downstream of PTEN, and that restoration of forkhead function might suppress tumorigenesis in PTEN-deficient tumor cells.

Materials and Methods

Plasmids. pCD19, pSG5L, pSG5L-HA-PTEN, pSG5L-HA-PTEN;G129R, pSG5L-HA-PTEN;G129E, pSG5L-HA-PTEN;1-353, pBABE-puroL, pBABE-puroL-HA-PTEN; pBABE-

puroL-HA-PTEN;G129R and pGL3-promoter (Promega) were described previously (54, 58, 69). pcDNA3-Flag-FKHR, pcDNA3-Flag-FKHR;H215, pcDNA3-Flag-FKHR;AAA, and pGL2promoter-3xIRS were gifts of E.Tang, F. Barr and K. Guan (67). The inserts from pcDNA3-Flag-FKHR or the mutant derivatives, restricted with BamHI and XbaI, were ligated to the vector from similarly restricted pcDNA3-GFP to give pcDNA3-GFP-FKHR, pcDNA3-GFP-FKHR;H215R and pcDNA3-GFP-FKHR;AAA. The oligonucleotides 5-'GCGCGGATCCATGGCCGAGGCGCCTCAGGTG3' , and 5'-CGCGCTCGAGGAATTCTCAGCCTGACACCCAGCTATG-3', were used to PCR amplify the FKHR cDNAs. The PCR products, restricted with BamHI and XhoI, were ligated to similarly restricted pBABE-puroL to give pBABE-puroL-FKHR, pBABE-puroL-FKHR;H215R, and pBABE-puroL-FKHR;AAA. The oligonucleotides 5'-GCGCGCTAGCGTGACAGAGTGAGACTCTGTCTCTATTTAAATAAATAAGTAAATAAA TAAAC-3' and 5'-GGGGAGATCTGCTTTGTATTTCACAATGTTTTCATTTTCATTGTTTGCCCAGTTTATTT ATTT-3' containing the forkhead site of the FasL promoter, were phosphorylated, annealed and ligated to pGL3-promotor restricted with BglII and NheI to give pGL3-promoter-FasL. This plasmid was subsequently restricted with BglII and HindIII, blunted and ligated to remove the SV40 promoter and give pGL3-FasL. pAdTrack -CMV and pAdEasy-1 were the gifts of B. Vogelstein and K. Polyak (29). The insert from pcDNA3-Flag-FKHR;AAA liberated by

restriction with XbaI and partial digestion with HindIII, was ligated to similarly restricted pAdTrack-CMV vector to give pAd-FKHR;AAA.

Cell lines, cell culture, transfection and MTS assay. LNCaP cells were maintained in RPMI 1640 containing 10% Fetal calf serum (FCS) (HyClone), penicillin and streptomycin (PS), 2.5 gm/L glucose and 10mM Hepes, 1mM sodium pyruvate, and 2 mM L-glutamine at 37°C in a humidified 5% CO_2 atmosphere. DU-145 cells were maintained in DMEM containing 10% FCS and PS at 37°C in a humidified 10% CO_2 atmosphere. ACHN, 786-O, and U2-OS cells were maintained as previously described (54). Phoenix-ampho (ϕX-A) cells were maintained in DMEM containing 10% FC and PS, at 37°C in a humidified 10% CO_2 atmosphere. 786-O cells were transfected using Fugene reagent (Boehringer-Mannheim) as previously described (71). U2-OS, ACHN, ϕX-A cells were transfected by the BBS calcium-phosphate method as previously described (11, 58).

LNCaP and 786-O cell viability was assayed using the Cell Titer 96 AQueous non-Radioactive Cell Proliferation Assay (Promega) according to the manufacturer's protocol. Briefly, cells were detached with trypsin and collected in 10 ml of complete media. 100 µl of cells was aliquoted in triplicate into 96 well plates. Twenty µl of a 1:20 dilution of phenazine methosulfate (PMS) in {3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt} (MTS) reagent was added to each well. The plate was

incubated at 37°C at 10% CO_2 for 15 min. Formazan product was detected by measuring the absorbance at 490 nm.

Antibodies, immunoblotting, protein extraction, cell fractionation. Anti-PTEN(C54) (54), anti-HA (BabCo), anti-FKHRL1, anti-phospho-FKHRL1 (Upstate Biotechnology), anti-GSK3 (New England Biolabs, NEB), anti-phospho-GSK3 (NEB), anti-phospho-Akt (NEB), anti-Akt (NEB), anti-p27 (Transduction Laboratories), anti-$p70^{S6K}$ (Santa Cruz Biotechnology) and 245 anti-RB (Pharmingen) antibodies were used at a dilution of 1:1000. M5 anti-flag antibody (Sigma) was used at 10μg/ml. Anti-p21 (Transduction Laboratories) was used at 1:500. Anti-tubulin (ICN) was used at 1:2000. Anti-GAPDH (Biodesign International) was used at 1:5000.

Cell lysates were prepared and immunoblots performed as previously described (71).

786-O and ACHN cells were fractionated by swelling for 10 min in RBS buffer (10 mM Hepes, pH 7.2, 10 mM NaCl, and 1.5 mM $MgCl_2$) containing leupeptin 5μg/ml, aprotinin 2 μg/ml, PMSF 50 μg/ml, NaF 5 mM, and NaOrthovanadate 0.5 mM. Cells were disrupted by 60 (786-O) or 10 (ACHN) manual strokes of a dounce homogenizer. Nuclei were pelleted by centrifugation at 2700 RPM's for 5 min, washed three times in RBS, and lysed in RIPA buffer (10 mM $NaPO_4$, 150 mM NaCl, 1% NP-40, 0.1% DOC, 0.1% SDS). Cytoplasmic proteins were precipitated with 10% Trichloracetic acid, washed with 80% acetone, washed with ddH_2O, and solubilized in 1X protein sample buffer.

FACS/Cell-cycle analysis. Cell cycle analysis was performed as previously described (58, 71). Briefly, 786-O cells grown on p100 plates were transfected with 4 μg of pCD19 plasmid and the amounts of either pSG5 or pcDNA3 expression plasmid indicated in the figure legends. Forty-eight h after transfection, cells were harvested, stained with fluorescein isothiocyanate-conjugated anti-CD19 antibody and propidium iodide and analyzed by two-color FACS (Beckton-Dickinson) .

Retrovirus production and infection. Amphotrophic retroviral supernatants were produced as previously described (52). Briefly, ϕX-ampho cells, split 1:4 the previous day, were transfected with 25 μg of the indicated pBABEpuroL plasmid DNA. After 16 h the media was changed and the cells incubated in 10% CO_2 incubator for 48 h. The media was harvested and stored at -70°C until needed. 786-O and LNCaP cells were incubated with 5ml of thawed viral supernatant containing 5 μg/ml polybrene (hexadimethine bromide) (Sigma H9268) and incubated at 37°C for 4 h. Five ml of complete media was added and the cells were maintained for 40 h in standard growth conditions after which the media was changed to complete media supplemented with 2 μg/ml puromycin. Drug resistant cells were selected and harvested after 72 h. Typically, 85% of LNCaP or 786-O cells infected with the pBABE-puroL retrovirus were drug resistant.

Adenovirus Production and Infection. Recombinant FKHR;AAA adenovirus was generated as previously described (29). Briefly, pAdTrack-CMV and pAd-FKHR;AAA were

linearized and individually co-transformed into electrocompetent BJ5183 cells (Quantum Biotechnologies) along with pAdEasy-1. Next, recombinant adenoviral DNA, isolated from kanamycin resistant colonies, was amplified in Top10 cells (Invitrogen), purified by $CsCl_2$ density gradient centrifugation, linearized with Pac1 and transfected into 293 cells with Lipofectamine (Life Technologies). After 7-10 days, packaged virus collected and used to infect 20 p150 plates of 293 cells. The amplified virus was isolated by freeze-thaw extraction, purified by $CsCl_2$ density gradient centrifugation and titered by lysis of 293 cells. LNCaP and 786-O cells were infected with Ad-vector at 50 moi and Ad-FKHR;AAA at 100 moi.

Reporter assays. Transfections for reporter assays were carried out in 6 or 24 well plates. Thirty-six h after transfection, cells were lysed in 1X Reporter Lysis Buffer according to the manufacturer's protocol (Promega). Cleared lysates were used in luciferase and ß-galactosidase assays as described previously (58). Relative light units were normalized to ß-galatosidase activity. The fold activation was obtained by dividing corrected luciferase values by the corrected luciferase value obtained in the presence of the vector and reporter plasmids alone.

Real-time quantitative polymerase chain reaction. RNA was prepared using the RNeasy RNA isolation kit (Qiagen) according to the manufacturer's protocol and included DNAse treatment. One µg total RNA was reverse-transcribed at 42°C for 45 min in a 20 µl reaction mixture containing 250 µM each dNTP, 20U of RNase inhibitor, 50U of MuLV Reverse Transcriptase (RT), 2.5 µM random hexamers, and 1X RT buffer (1.5 mM $MgCl_2$), and then

denatured at 99°C for 5 min. A RT minus reaction was performed for each sample. Specific primers and fluorogenic probe for human p27 (Fw: 5' GCAATGCGCAGGAATAAGGA 3'; Rev: 5' TCCACAGAACCGGCATTTG 3'; Probe: 5' CGACCTGCAACCGACGATTCTTCTACTCA 3') were designed using Primer Express 1.0 Software. Amplification of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used to standardize the amount of RNA in each reaction (Taqman GAPDH Control Reagents). PCR was performed using an ABI PRISM 7700 Sequence Detector. The Taqman® PCR Core Reagent Kit was used according to the manufacturer's protocol with the following modifications: dUTP was replaced by dTTP and incubation with AmpErase was omitted. PCR reactions each contained 1 µl cDNA (equivalent to 50 ng template RNA), 2.5 U of AmpliTaq Gold, 100 nM oligonucleotide primers and fluorogenic probe in a volume of 50 µl. Amplifications consisted of 60 cycles of 94 C° for 45 sec, 58 C° for 45 sec, and 72 C° for 1 min. All reagents for real-time PCR were purchased from Perkin-Elmer Applied Biosystems.

In each experiment, additional reactions with 7 serial two-fold dilutions of 786-O cDNA as template were performed with each set of primers and probes on the same 96 well plate to generate standard curves, which related the threshold cycle (C_T) to the log input amount of template. All samples were amplified in triplicates. The relative amount of p27 transcripts in each sample was determined by using the standard curve method and by normalizing for GAPDH mRNA expression levels, as previously described (1, 21).

Protein half-life determination by cycloheximide treatment. Twenty h after adenoviral infection, 786-O cells were treated with 25μg/ml of cycloheximide. At the indicated times, cells were washed, scrape harvested into 500μl of PBS, pelleted by centrifugation at 400 x g for 5 min and stored at –70°C. Cell extracts were prepared as described above and immunoblotted with the indicated antibodies. Multiple exposures were obtained and then digitized using a Scanmaker III flatbed scanner. The resulting immunoblot signals were quantified using ImageQuant software (Molecular Dynamics). Only radiographs where the peak quantification showed non-saturating signals were used. The half-life was calculated from exponential curve fits to the data plotted in log-linear fashion, as previously described (71).

Results

FKHRL1 protein levels and phosphorylation are deregulated in the absence of PTEN. A survey of the activation-state of downstream targets of Akt was undertaken using antibodies against specific phosphopeptides. Two pairs of cell lines were used, ACHN and 786-O renal carcinoma cells and DU145 and LNCaP prostate carcinoma cells. ACHN and DU145 both retain wild-type PTEN alleles and express an intact PTEN protein while 786-O and LNCaP cells fail to express any full-length PTEN protein (54) (Fig. 1). Whole cell extracts were prepared from serum-starved cells or from starved cells that were stimulated with serum. As previously shown, in these *PTEN-/-* cells the phosphorylated and activated form of Akt is overabundant (54), (Fig. 1). Extracts were immunoblotted with antibodies that detect phosphorylation of

GSK3 and FKHRL1. In the absence of serum, deregulation of GSK3-α phosphorylation was noted in the two PTEN null cell lines (Fig. 1). Immunoblotting also demonstrated a marked in crease in phosphorylated FKHRL1 in the PTEN null cell lines, however the total amount of FKHRL1 was also elevated in these cells. While both endogenous AFX and FKHR were detected in all of these cells (data not shown), phosphospecific antibodies were, in our hands, incapable of recognizing endogenous phosphorylated AFX or FKHR. In contrast to the results obtained with FKHRL1 and GSK3, phosphorylated Bad was not detected in these cells and $p70^{S6K}$ was not consistently hyperphosphorylated in a manner that reflected loss of PTEN (data not shown and Fig. 1).

FKHR localization is constitutively cytoplasmic in PTEN -/- cells. Previous data showed that Akt-dependent inhibition of FKHR or FKHRL1 is mediated, at least in part, by phosphorylation-dependent localization of these transcription factors to the cytoplasm (5, 6). These considerations and the data in Fig. 1 led us to ask whether forkhead factors might be aberrantly localized in PTEN null cells. To this end 786-O and ACHN cells were fractionated into cytoplasmic and nuclear fractions. Anti-FKHRL1 immunoblotting demonstrated that FKHRL1 was indeed cytoplasmic in PTEN null 786-O cells, while in ACHN cells, FKHRL1 was primarily localized to the nucleus (Fig. 2C). As controls for fractionation, immunoblotting demonstrated that ß-tubulin was found in the cytoplasm and the Retinoblastoma protein (pRB) was found in the nucleus (Fig. 2C).

In order to examine localization of Forkhead factors in living cells, plasmids encoding green fluorescent protein (GFP)-FKHR fusion proteins were introduced into cells containing or lacking PTEN. Here, we chose to use FKHR as a representative of the class of forkhead transcription factors that include FKHR, FKHRL1, and AFX. After 24 h, the localization of GFP-FKHR in living cells was determined by direct visualization using fluorescence microscopy. In cells that have PTEN, GFP-FKHR was found localized primarily in the nucleus (ACHN) or in both the nucleus and cytoplasm (U2-OS). In contrast, GFP-FKHR was localized exclusively in the cytoplasm in cells lacking PTEN (786-O and LNCaP) (Fig. 2A). These data were quantified by manual counting of cells (Fig. 2B). In contrast, a FKHR mutant (FKHR;AAA) lacking the three Akt phosphoacceptor sites (T24A, S256A, S319A) (67), was found primarily in the nucleus of PTEN null cells (Fig. 2A). These data suggest that in PTEN null cells, FKHR is mislocalized to the cytoplasm due to persistent activation of the PI3K pathway and hence persistent FKHR phosphorylation.

PTEN expression relocalizes GFP-FKHR to the nucleus of PTEN null cells. In order to determine whether re-expression of PTEN protein could effect a change in the localization of GFP-FKHR, plasmids encoding either wild-type or mutant PTEN derivatives were transfected into both 786-O and LNCaP cells along with the plasmid encoding GFP-FKHR. In greater than 90% of LNCaP or 786-O cells co-transfected with PTEN;WT, GFP-FKHR was localized to nuclei (Fig. 3A, B and C). In contrast, GFP-FKHR remained cytoplasmic when co-produced

with either PTEN mutant (PTEN;G129R and PTEN;G129E) (Fig. 3A, B and C). PTEN;G129E retains protein, but not lipid phosphatase activity whereas PTEN;G129R lacks both these activities (22, 44, 54). Thus, PTEN protein phosphatase activity is not sufficient for the induction of nuclear localization of GFP-FKHR. PTEN;1-353 is a truncated form of PTEN, that retains lipid and protein phosphatase activity, and can inhibit cell-cycle progression and Akt kinase activity comparably to wild-type PTEN (Fig. 3A, B and C) (S. Ramaswamy and W. R. Sellers, unpublished data, (33, 71)). In keeping with these data, expression of PTEN;1-353 led to the nuclear accumulation of GFP-FKHR;WT. Together these data suggest that FKHR is aberrantly localized in PTEN null cells and that reconstitution of PTEN lipid phosphatase activity is sufficient for localizing FKHR to the nucleus.

FKHR transcriptional activity is defective in PTEN null cells. FKHR can activate transcription from a minimal promoter element contained within the IGFBP-1 promoter (28, 67). Likewise, FKHRL1 can activate transcription from a sequence derived from the FasL promoter (6)). The localization data obtained using GFP-FKHR fusion proteins suggested that FKHR might not activate transcription in a PTEN null cell. To test this, *PTEN*+/+ ACHN and U2-OS cell were transfected with a luciferase reporter plasmid containing a 3xIRS element, or a FasL promoter element, along with a plasmid encoding Flag-tagged FKHR. In these cells, FKHR transfection resulted in a dose-dependent increase in transcription (Fig. 4A and B, and data not shown). In these same cells FKHR;H215R, harboring a point mutation in the DNA-binding

domain, had no effect (data not shown). In contrast to these results transfection of wild-type FKHR in the PTEN null 786-O and LNCaP cells, did not activate transcription from either the 3xIRS or FasL promoter elements (Fig. 4C and D, and data not shown). These data demonstrate that the ability of FKHR to activate transcription is defective in PTEN null cells. Note that neither reporter used in these experiments was capable of assaying endogenous forkhead activity. Specifically, in the absence of exogenous FKHR, when these reporters were compared to the same reporters lacking an intact Forkhead DNA binding, there was no significant difference in overall transcriptional activation (data not shown). This presumably indicates that other elements in these synthetic promoters contributes to the relatively high level of basal activity.

While wild-type FKHR failed to activate transcription in these cells, the phosphorylation site mutant FKHR;AAA was capable of activating transcription in the PTEN null cells (data not shown). We next asked whether PTEN, as an antagonist of PI3K/Akt signaling, could rescue FKHR transcriptional activation. To this end, 786-O cells were co-transfected with the FasL promoter-luciferase reporter plasmid along with either empty vector, or wild-type FKHR. In keeping with the data in Fig. 4, wild-type FKHR did not activate transcription from this promoter (Fig. 5A and B). Likewise, co-transfection of wild-type FKHR with plasmids encoding the PTEN mutants G129R or G129E failed to activate the FasL promoter (Fig. 5A). In contrast, co-transfection of plasmids encoding either PTEN;WT or PTEN;1-353 rescued transcriptional activity (Fig. 5A). As a control, production of PTEN;WT along with the DNA-binding defective

mutant FKHR;H215R, had no effect on transcription (Fig. 5A) despite the fact that PTEN relocalizes the GFP-FKHR;H215R mutant efficiently to the nucleus (Fig. 3A and D). In order to ask whether these observations held with other FKHR responsive reporters, we performed similar experiments using the 3xIRS-luciferase reporter plasmid (Fig. 5B and 5D). Here, FKHR again was incapable of activating transcription when overexpressed. Co-transfection of FKHR along with either PTEN;WT or PTEN;1-353 restored FKHR-dependent activation while, PTEN;G129R and PTEN;G129E did not. Finally, PTEN restored dose-dependent transcriptional activity of FKHR;WT when measured on both the FasL promoter and the 3xIRS promoter, while PTEN;G129R had no effect at the highest doses of FKHR tested (Fig. 5C and 5D). Taken together the above data suggest that PTEN allows for appropriate localization of FKHR and for appropriate transcriptional function.

Activated FKHR induces cell-death in LNCaP. Certain PTEN null cells, such as PTEN-/- MEFs are resistant to apoptotic stimuli (63, 65) and PTEN reconstitution to, or treatment with PI3K inhibitors of certain PTEN null tumor cells (e.g. UMG-251 or LNCaP), results in the induction of cell-death that is, at least in part, mediated through apoptosis (10, 15, 16, 36). Likewise, FKHRL1 and FKHR can both induce apoptosis (6, 67). Thus, we next asked whether FKHR or the FKHR;AAA mutant could induce cell death in PTEN-null LNCaP or 786-O cells. To test this, PTEN null 786-O or LNCaP cells were incubated with culture supernatants containing amphotrophic retroviruses encoding PTEN;WT, PTEN;G129R, FKHR;WT and

FKHR;AAA and were then selected with puromycin. In keeping with previously reported results (15, 44), PTEN induced cell death in LNCaP cells and completely suppressed the emergence of puromycin resistant cells (Fig. 6A and C). On the other hand, infection with retrovirus producing PTEN;G129R had no effect on cell viability. Cells infected with FKHR;WT were more prone to cell death than vector infected controls, however puromycin resistant populations expressing FKHR;WT were obtained (data not shown). In comparison, infection with retrovirus producing FKHR;AAA, like PTEN, led to marked suppression of cell viability and completely suppressed the emergence of puromycin resistant clones. Thus, the activated form of FKHR complimented PTEN deficiency in these cells.

In contrast to the results obtained in LNCaP cells, retroviral transduction of PTEN into 786-O cells, did not induce cell death (Fig. 6A, B and D). 786-O cultures were likewise infected with viruses leading to the production of FKHR or FKHR;AAA. Here, surprisingly, FKHR and FKHR;AAA had little overall effect on cell viability (Fig. 6A and D). Furthermore, puromycin-resistant polyclonal lines expressing these proteins were derived (Fig. 6B). Thus, activated FKHR can induce apoptosis in a cell line in which PTEN induces apoptosis, but does not induce apoptosis in a cell line immune to PTEN induced apoptosis.

FKHR induces a cell-cycle block in PTEN null cells. In U87-MG and 768-O cells, reintroduction of PTEN by adenoviral infection or transient transfection induces a cell cycle arrest in G1 rather than apoptosis (22, 36, 54). One possibility, among many, for the lack of

apoptosis in these cells is that additional genetic alterations in these cells render PTEN incapable of inducing apoptosis. If FKHR is a critical downstream activator of apoptosis in these cells, perhaps this putative defect is a defect in FKHR function. If so, this might account for the lack of effect of the FKHR;AAA mutant in the cell-death assay performed in 786-O cells (Fig. 6A). Alternatively, FKHR or other Forkhead factors might function in both the apoptotic and cell-cycle function of the PI3K/PTEN/Akt pathway. To test this hypothesis, a transient cell-cycle assay was utilized. 786-O cells were transiently transfected with a plasmid encoding the cell-surface marker CD19 along with plasmids encoding PTEN;WT or PTEN;G129R. PTEN;WT, but not PTEN;G129R induced a modest cell-cycle block. While, FKHR;WT had a minimal effect on the G1 population, FKHR;AAA induced a robust G1 arrest (Fig. 7A), whereas FKHR;H215R did not. Thus, activated FKHR can compliment the loss of PTEN in 786-O cells. We next asked whether PTEN could "rescue" the apparent defect in FKHR;WT mediated cell-cycle arrest. In keeping with the ability of PTEN to relocalize FKHR and to restore transcriptional activation, co-transfection of PTEN;WT along with FKHR;WT led to an increase in the G1 population that was comparable to that induced by the FKHR;AAA mutant (Fig. 7B). These data suggest that restoration of functional FKHR to these cells, by co-transfection of PTEN or by rendering FKHR immune to Akt phosphorylation, is sufficient to arrest PTEN null cells in G1.

Loss of PTEN in cells leads to a reduction in p27 protein levels (34, 65). Thus, in order to begin to characterize the G1 arrest induced by FKHR;AAA we first examined p27 protein

levels. To investigate this, 786-O cells were transiently transfected with a plasmid encoding the cell-surface marker CD19 along with the vector plasmid or with plasmids encoding PTEN;WT, FKHR;WT or FKHR;AAA. The CD19+ and hence transfected cells were collected on anti-CD19 coated magnetic beads. Protein extracts were prepared and immunoblotted with antisera specific for p27. Here, wild-type PTEN and constitutively active FKHR;AAA, both induced p27 protein (Fig. 7C). In this cell line, immunoblots for p27 consistently show a doublet that is recognized by multiple independent anti-p27 antisera (data not shown). Anti-GAPDH immunoblotting served to confirm equivalent protein loading. In addition, while PTEN induced a modest increase in p27, wild-type FKHR co-transfected along with wild-type PTEN induced p27 levels comparably to FKHR;AAA (Fig. 7C). These data were confirmed and extended using retroviral delivery of the FKHR;AAA to 786-O cells and with adenoviral delivery of FKHR;AAA (Fig. 8). While p27 was again induced by FKHR;AAA, p21 was not (Fig. 7D). These data suggest that p27 is a specific downstream target of FKHR. Finally, infection of 786-O, but not infection of LNCaP cells, with adenovirus directing the expression of FKHR;AAA (Ad-FKHR;AAA) induced p27 protein.

While our data and the genetic evidence in *C.elegans* suggests that Forkhead factors are critical downstream targets of PTEN, it is possible that our results reflect not a downstream effect of FKHR, but rather a negative regulation of Akt by FKHR, perhaps through feedback inhibition. To ask whether such a mechanism might account for FKHR actions in these cells, we examined the state of phosphorylation and hence activation of Akt. The immunoblots described

above were stripped and re-probed with antisera specific to the Serine 473 phosphorylation on Akt. Here, in keeping with previously published data, production of wild-type PTEN led to an ablation of phospho-Akt (data not shown) while FKHR;AAA production was associated with a modest increase in phosphorylated Akt, while total Akt remained unchanged (Fig. 7C and data not shown). These data suggest that feedback inhibition of Akt is not the mechanism by which FKHR promotes either apoptosis or a G1 arrest.

FKHR;AAA induces p27 mRNA and prolongs the p27 protein half-life. In order to begin to address the mechanism through which FKHR regulates p27, p27 mRNA levels were determined in 786-O cells following adenoviral infection with Ad-FKHR;AAA. At 24 h, cells infected with Ad-FKHR;AAA demonstrated a modest 1.3 - 1.5 fold induction of the p27 mRNA when compared to those cells infected with Ad-vector (Fig. 8B). Consistent with these results, we have found Ad-PTEN induces a 1.8 fold induction in p27 mRNA (S. Ramaswamy, S. Signoretti, M. Loda and W.R. Sellers, unpublished data). Next, 786-O cells were again infected with Ad-vector or Ad-FKHR;AAA. Twenty-four h after infection cells were treated continuously with cycloheximide (25 μg/ml). At specific time points, the p27 protein level was determined by immunoblotting in both the Ad-vector and Ad-FKHR;AAA infected cells. Here, we found that the protein half-life was increased from 123 minutes to 329 minutes in the Ad-FKHR;AAA infection. Thus FKHR;AAA induces a modest change in the p27 mRNA level and a significant increase in the p27 protein half-life.

Discussion

Our data show that localization and transcriptional activity of FKHR is aberrant in PTEN null cells. Reconstitution of wild-type PTEN, but not lipid phosphatase inactive mutants, restores both localization and transcriptional activation of FKHR in these cells. While wild-type FKHR is relatively inactive in PTEN null cells, a phosphosite mutant of FKHR (FKHR;AAA), that is no longer phosphorylated by Akt, can still localize to the nucleus and activate transcription in such cells. This mutant induces cell death in a cell line susceptible to PTEN-mediated cell death. Surprisingly, it does not induce apoptosis, but rather induces a G1 arrest in cells that likewise arrest with wild-type PTEN. Together the data derived from the cell death assays and cell cycle arrest assays support the notion that an intact and active FKHR protein is capable of carrying out PTEN function in its absence. That is, activated FKHR compliments the loss of PTEN in two different functional assays. These data support the idea that FKHR is sufficient for PTEN function in cells. Finally, previous data have shown that PTEN null cells have low levels of p27 and that reintroduction of PTEN up regulates p27 levels (34, 65). We find that FKHR;AAA dramatically induces p27 levels in PTEN null cells. These data suggest that the finding of aberrant p27 levels in the absence of PTEN might arise as a consequence of the lack of FKHR function in such cells. In keeping with these data, Medema et. al. recently reported similar data which demonstrated a role for Forkhead factors as regulators of cell-cycle progression and using defined genetic cells showed that such regulation does indeed depend upon the induction of p27 (41).

The PI3K/Akt pathway is a long known oncogenic signaling pathway (76). Cell-survival and cell proliferation have been linked to this pathway in multiple systems. For example, IL-3 dependent cell lines require Akt for survival as do cells in which anoikis is blocked by Ras activation (18, 30, 62). On the other hand, expression of activated PI3K in the absence of serum can induce DNA synthesis (31). Furthermore, PTEN is capable of inducing apoptosis or a cell cycle arrest and loss of PTEN in primary cells leads to either excessive proliferation or defects in apoptosis. In mammalian cell-based assays, a diverse group of substrates has been linked to Akt activation. In *C.elegans*, on the other hand, the insulin/PI3K/Akt signaling pathway that regulates aging, while conserved with mammalian cells, has thus far yielded only the forkhead homologue, *daf-16* as a downstream target (47). It is possible that the deregulated activity of multiple Akt substrates contribute to the neoplastic properties inherent to a PTEN null tumor cell, and that certain substrates might individually contribute to the regulation of apoptosis and cell proliferation. Our data, however, support the notion that the pathway linking PI3K and PTEN to transformation of mammalian cells is essentially identical to the pathway regulating aging in *C.elegans* . This pathway is comprised of a receptor tyrosine kinase such as IGF-IR (*daf-2*), PI3K (*age1*), Akt-1 (*akt1*) and Akt-2 (*akt2*), PDK-1 (*pdk1*), PTEN (*daf-18*) and the daf-16 homologues (FKHR, FKHRL1, AFX) (24, 26, 43, 47, 48, 50, 51, 56).

It is interesting to note that elements of this pathway that are linked genetically in *C.elegans* are the same elements of the pathway that have been associated with genetic alterations in human tumors. The PI3KCA gene is amplified in ovarian cancer and is the product

of a retroviral oncogene(2, 60). Akt-1 and Akt-2 are amplified in a limited number of tumors, and Akt-1 is the cellular homologue of v-akt (3, 4, 12, 57). Finally, *PTEN* is widely mutated in cancer, and FKHR has been the target of translocation in rhabdomyosarcoma. Interestingly, in this tumor, two different translocations give rise to fusion proteins PAX3-FKHR or PAX7-FKHR (17, 23, 59). Our data support the notion that FKHR could act as a tumor suppressor, thus one untested possibility is that these translocations might produce chimeric proteins that could act in a dominant negative manner to inactivate FKHR function.

The notion that a transcription factor might induce a G1 arrest or induce cell death is not new. Indeed, this is precisely the case for p53. The parallels between these pathways are striking. p53 receives signals that reflect the state of the genome (DNA damage), at least in part, from a PI3K family member ATM. This signal is transmitted perhaps through phosphorylation of p53. p53 can then enact a G1 arrest through transcriptional regulation of p21. p53 induces apoptotic cell-death through both transcription dependent and independent mechanisms. FKHR on the other hand receives signals primarily from the environment external to the cell. These signals are transmitted through a type I PI3K and result in the phosphorylation of FKHR and its subsequent inactivation. In its active state FKHR, can promote a G1 arrest through the induction of p27 and can induce apoptosis perhaps through regulation of Fas signaling or through regulation of FasL itself.

How does FKHR regulate p27? p27 is primarily regulated post-transcriptionally, both through ubiquitin-mediated proteolysis or through translation controls. There is limited

information to suggest that transcriptional regulation of p27 is important. Furthermore, PTEN did not alter p27 mRNA levels (34). On the other hand, Medema et. al. and have demonstrated activation of the p27 promoter by AFX, and we have shown that both wild-type PTEN and wild-type FKHR, but not mutant controls, were capable of inducing activation of the p27 promoter (data not shown)(41). In addition, Medema et. al. reported a modest induction in p27 mRNA levels. We have also seen a modest 1.3-1.5 fold induction in mRNA upon adenoviral expression of FKHR;AAA (Fig. 8B) and upon adenoviral expression of PTEN. In addition, however, the p27 protein half-life is also significantly prolonged. Here, it is possible that a modest increase in p27 levels induced through transcriptional, might lead to inhibition of cyclin-dependent kinase activity, followed by a subsequent decrease in p27 phosphorylation, and then a change in the p27 protein half-life. As this process involves a catalytic mechanism, a small change in p27 mRNA levels could lead to a large difference in protein half-life. For example, increase in transcription of p27 could alter the balance between the two proposed complexes of p27 and Cyclin E/cdk2, one inhibitory and one in which p27 is degraded (61, 73). Alternatively, it is possible that FKHR;AAA directly alters or regulates components of the p27 degradation apparatus. Specifically, it will be of interest to know whether forkhead factors can alter levels of any of the components of the SCF complex.

The mechanism that underlies FKHR induction of apoptosis is likewise not yet clear. FKHRL1 can regulate the FasL promoter suggesting that these transcription factors might directly regulate the levels of this death effector (6). In keeping with this notion, *PTEN*+/- mice

develop an autoimmune lymphoid hyperplasia syndrome that phenocopies mutations in the murine Fas gene (19, 75). On the other hand, cells from the *PTEN*+/- animals did not demonstrate defects in FasL or Fas, but were rather defective in the apoptotic response to Fas (19). In either case, it would appear that PTEN- and by extension FKHR-mediated apoptosis likely involves the Fas pathway.

Finally, our data support the notion that, as is the case in *C.elegans*, signaling pathways might be more linear, at least with respect to transformation, than is commonly suspected. This would lead one to further suspect that PTEN null cells might be particularly sensitive to inhibitors directed against members of this pathway, and if true such dependence would bode well for the future success of therapeutics aimed at intervening in PI3K signaling.

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Figure Legends

Fig. 1. Immunoblot detection of Akt substrates in PTEN null cells

Whole cell extracts were prepared from the indicated serum-starved or serum-stimulated cells and separated by gel electrophoresis. Separated proteins were transferred to nitrocellulose. Membranes were incubated with the indicated immune reagents and bound antibody was detected by enhanced chemiluminescence. For GSK-3 serum stimulation was carried out for 10 min, while in the remaining blots, cells were stimulated for 90 min. In certain instances the lane order was change for the purpose of clarity.

Fig. 2. FKHR is mislocalized in PTEN null cells

(A) Fluorescence microscopy of GFP-FKHR in PTEN plus and PTEN null cells.

The indicated cells were transiently transfected, as described in the methods, with a plasmid encoding GFP-FKHR or GFP-FKHR(AAA). Twenty-four h after transfection, GFP-FKHR was detected by fluorescence microscopy in living cells.

(B) Quantification of the results obtained with GFP-FKHR as shown in A. The percentage of cells with nuclear localization of GFP-FKHR was determined by manual counting of GFP positive nuclei. Data shown are the mean and standard error of duplicate experiments and are representative of two independent experiments.

(C) Localization of FKHRL1 in 786-O cells and ACHN cells.

786-O and ACHN cells were fractionated into cytoplasmic and nuclear compartments by hypotonic lysis and dounce homogenization. Equivalent cell fractions were loaded on the gel and immunoblotted with anti-FKHRL1, anti-ß-tubulin and anti-pRB antibodies as indicated.

Fig. 3. Reconstitution of PTEN re-localizes GFP-FKHR to the nucleus

(A) The cellular localization of GFP-FKHR in 786-O and LNCaP cells. 786-O cells were transiently transfected with pCDNA3-GFP-FKHR along with pSG5L or pSG5L-PTEN, or plasmids encoding the indicated mutant derivatives. Similarly, LNCaP cells were transiently co-transfected with pCDNA3-GFP-FKHR, or GFP-FKHR;H215R along with pSG5L plasmid encoding the indicated PTEN cDNAs. Twenty-four h after transfection, GFP-FKHR was detected by fluorescence microscopy in living cells.

(B) Quantitation of the results from panel A (left). The percentage of cells with nuclear GFP-FKHR was determined as in Fig. 2B. The mean and standard error for experimental duplicates is shown and the results obtained are representative of the results obtained in three independent experiments

(C) Quantification of the results from panel A (middle). Data is shown as in B.

(D) Quantification of the results from panel A (right). Data is shown as in B.

Fig. 4. FKHR-dependent transcriptional activation is defective in PTEN null cells

(A and B) FKHR transactivates the 3xIRS promoter in ACHN and U2-OS cells.

ACHN and U2-OS cells were transiently co-transfected with a 3xIRS-luciferase reporter plasmids along with either pCDNA3 vector, or with the indicated amounts of pCDNA3-Flag-

FKHR. In each transfection a constant amount of pCMX-ßGal plasmid was included. Thirty-six h after transfection luciferase activity was determined as described in the methods. Fold activation was calculated by normalizing the measured light units by the measured ß-galactosidase activity and then normalizing to the activity of the 3xIRS-promoter luciferase construct when transfected with vector alone. Data shown is the mean and standard error of independent duplicate experiments and is representative of three independent experiments.

(C and D) FKHR fails to activate transcription in 786-O and LNCaP cells.

786-O and LNCaP cells were transiently co-transfected with a 3xIRS-luciferase reporter plasmid and either vector or pCDNA3-Flag-FKHR as in A and B. Data is shown as for panels A and B.

Fig. 5. Reconstitution of PTEN restores FKHR transcriptional activation

(A and B) Wild-type PTEN and PTEN;1-353 restore transcriptional activation of FKHR in 786-O cells. 786-O cells were transiently co-transfected with either the FasL promoter-luciferase reporter plasmid (A) or the 3xIRS promoter-luciferase reporter plasmid along with plasmids encoding the indicate proteins. Data is shown as in Fig. 4A.

(C and D) Wild-type PTEN, but not PTEN;G129R rescues dose-dependent FKHR transactivation. 786-O cells were co-transfected with either the FasL promoter-luciferase reporter plasmid (C) or the 3xIRS promoter luciferase plasmid along with plasmids encoding the indicated proteins. Data is shown as for Fig. 4A.

Fig. 6. FKHR induces cell death in PTEN null LNCaP, but not PTEN null 786-O cells.

(A) 786-O and LNCaP cells were infected with retroviruses directing the expression of the indicated proteins. Infected cells were then grown in the presence of puromycin and viable cells were photographed by light microscopy after 4 days of selection (100X).

(B) Protein expression in puromycin resistant populations of 786-O cells. Whole cell extracts were prepared from puromycin resistant 786-O cells infected as in A and immunoblotted with either anti-PTEN or anti-HA antibodies.

(C and D) MTS assays were performed, as described in the methods, to quantify the results shown in A. In order to normalize the results between the two cell lines, cell viability is expressed as a percentage of the vector controls. The data shown is the mean and standard error of independent duplicates and is representative of two independent infections.

Fig. 7. FKHR induces a G1 arrest in PTEN null 786-O cells

(A) FKHR;AAA induces a G1 arrest in PTEN null 786-O cells. 786-O cells were transiently transfected with pCD19 and plasmids directing the expression of the indicated proteins. Forty-eight h after transfection the cell-cycle distribution of the transfected cells was determined by combined FITC-anti-CD19 and propidium iodide staining. The data shown are the mean and standard error of replicates and are representative of two independent experiments.

(B) PTEN cooperates with FKHR to induce a cell-cycle arrest. 786-O cells were transfected with pCD19 and plasmids directing the expression of the indicated proteins. The data shown are as in panel A.

(C) PTEN and FKHR;AAA induce p27 in 786-O cells. 786-O cells were transiently transfected with a plasmid encoding the cell-surface marker CD19, along with either vector plasmid, or plasmids encoding the indicated proteins. Thirty-six h after transfection, cells were harvested by trypsinization, and collected on anti-CD19 coated magnetic beads. Seventy-five μg of whole cell extracts, derived from the isolated cells, were separated by gel-electrophoresis and immunoblotted with the indicated antibody reagents.

(D) FKHR;AAA induces p27, but does not induce p21. 786-O cells were infected with retroviruses encoding the indicated proteins and selected with puromycin. Following selection protein extracts were prepared and immunoblotted with the indicated antibody reagents.

Fig. 8. FKHR;AAA induces p27 mRNA and prolongs the p27 protein half-life.

(A) FKHR;AAA induces p27 in 786-O, but not LNCaP cells. 786-O and LNCaP cells were infected with either Ad-vector or Ad-FKHR;AAA as indicated. Thirty-six h after infection, protein extracts were prepared and immunoblotted with the indicated antibody reagents.

(B) FKHR;AAA induces p27 mRNA in 786-O cells. 786-O cells were infected with Ad-vector or Ad-FKHR;AAA as indicated. 24 h after infection cells were harvested, and mRNA was harvested and protein extracts were prepared from duplicate plates. p27 mRNA was measured by real-time quantitative PCR using a ABI PRISM 7700 Sequence Detector as described in the methods. Each sample was assayed in triplicate for both p27 mRNA and GAPDH mRNA. Fold induction of mRNA indicates the p27/GAPDH ratio of each sample normalized to the ratio

obtained with Ad-vector. The data obtained from two independent infections performed on separate days are shown.

(C) FKHR;AAA prolongs the half-life of the p27 protein. 786-O cells were infected with Ad-vector or Ad-FKHR;AAA as for panel B. Twenty-four h after infection, cycloheximide was added to a final concentration of 25 μg/ml. Cycloheximide was maintained for the entire course of the experiment. Protein extracts were prepared at the indicated time points and immunoblotted with the indicated antibody reagents.

(D) Calculation of the p27 protein half-life. Multiple radiographic exposures of the experiment shown in panel C were obtained (data not shown). Quantitation of the p27 signal intensity was obtained from exposures in which the signal was non-saturating for the entire time course. Signal intensities were normalized to the signal intensity obtained at the time zero. The percent signal remaining was plotted on a log-linear plot and a exponential curve fit applied. The calculated half-life is shown. These results are representative of two-independent experiments.

Figure 1

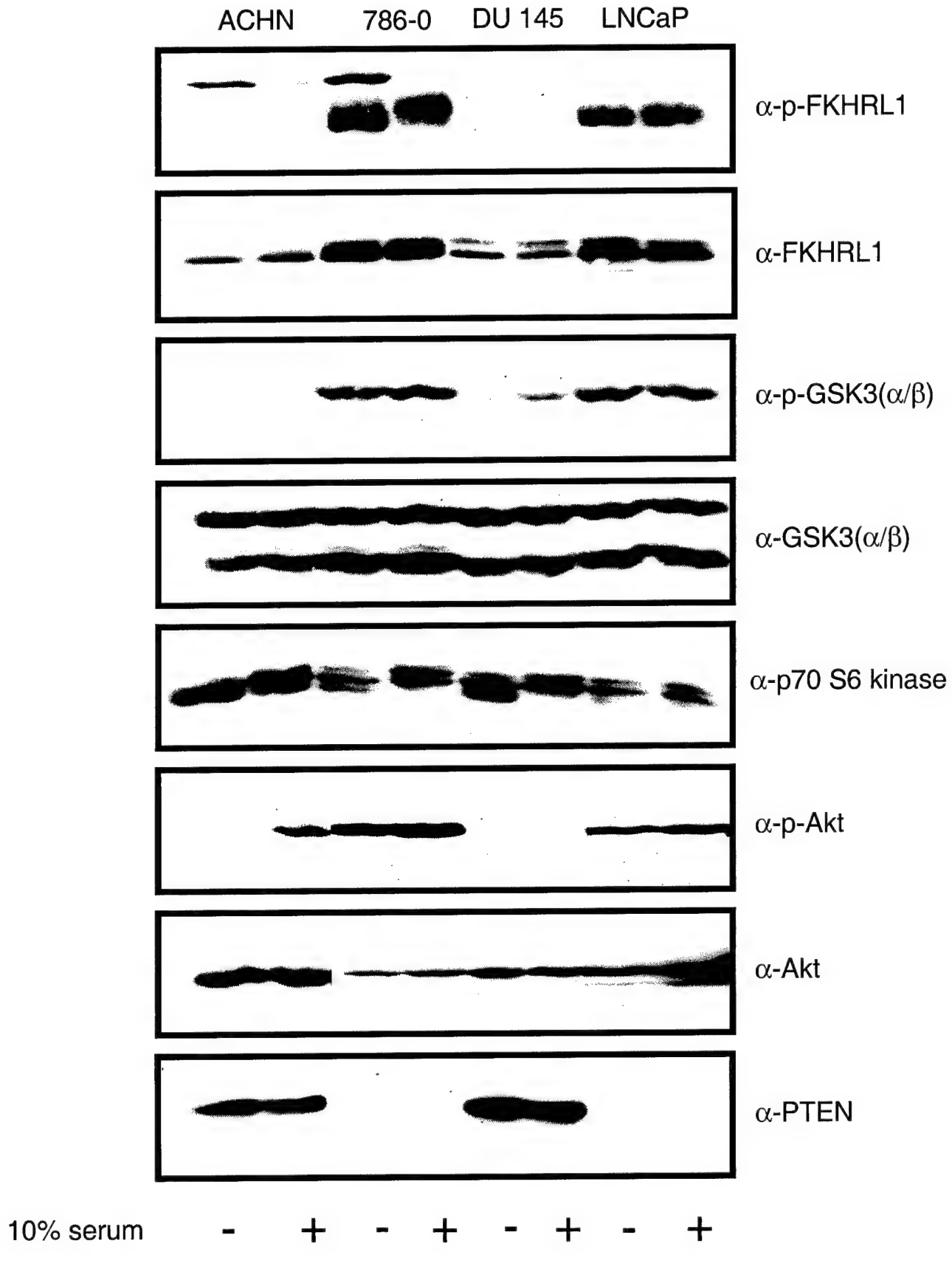

ACHN
786-0
DU 145
LNCaP
α-p-FKHRL1
α-FKHRL1
α-p-GSK3(α/β)
α-GSK3(α/β)
α-p70 S6 kinase
α-p-Akt
α-Akt
α-PTEN
10% serum
- + - + - + - +

Figure 2

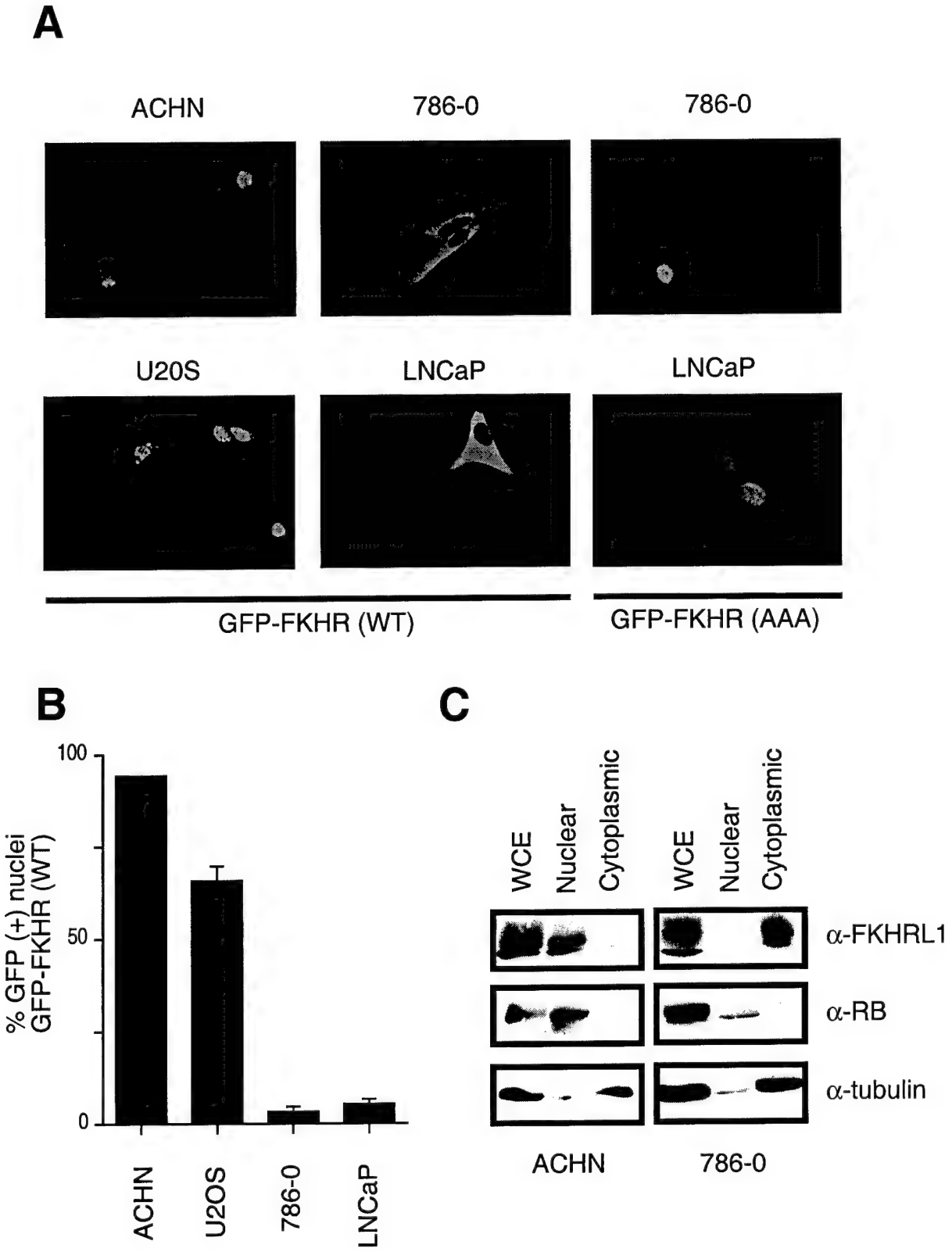

A
ACHN
786-0
786-0
U20S
LNCaP
LNCaP
GFP-FKHR (WT)
GFP-FKHR (AAA)
B
% GFP (+) nuclei GFP-FKHR (WT)
100
50
0
ACHN
U2OS
786-0
LNCaP
C
WCE
Nuclear
Cytoplasmic
WCE
Nuclear
Cytoplasmic
α-FKHRL1
α-RB
α-tubulin
ACHN
786-0

Figure 3

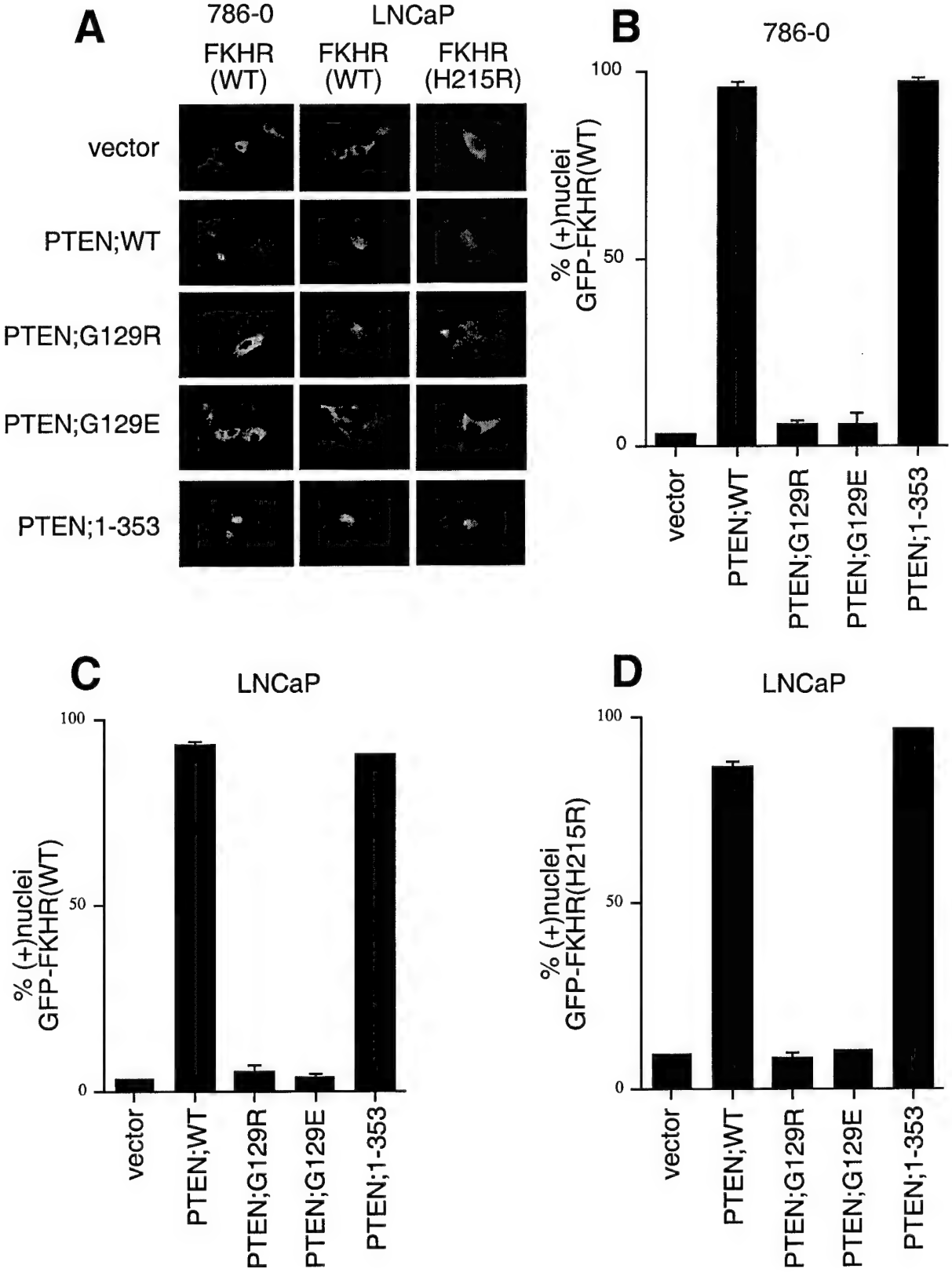

A
786-0
LNCaP
FKHR (WT)
FKHR (WT)
FKHR (H215R)
vector
PTEN;WT
PTEN;G129R
PTEN;G129E
PTEN;1-353
B
786-0
% (+)nuclei GFP-FKHR(WT)
100
50
0
vector
PTEN;WT
PTEN;G129R
PTEN;G129E
PTEN;1-353
C
LNCaP
% (+)nuclei GFP-FKHR(WT)
100
50
0
vector
PTEN;WT
PTEN;G129R
PTEN;G129E
PTEN;1-353
D
LNCaP
% (+)nuclei GFP-FKHR(H215R)
100
50
0
vector
PTEN;WT
PTEN;G129R
PTEN;G129E
PTEN;1-353

Figure 4

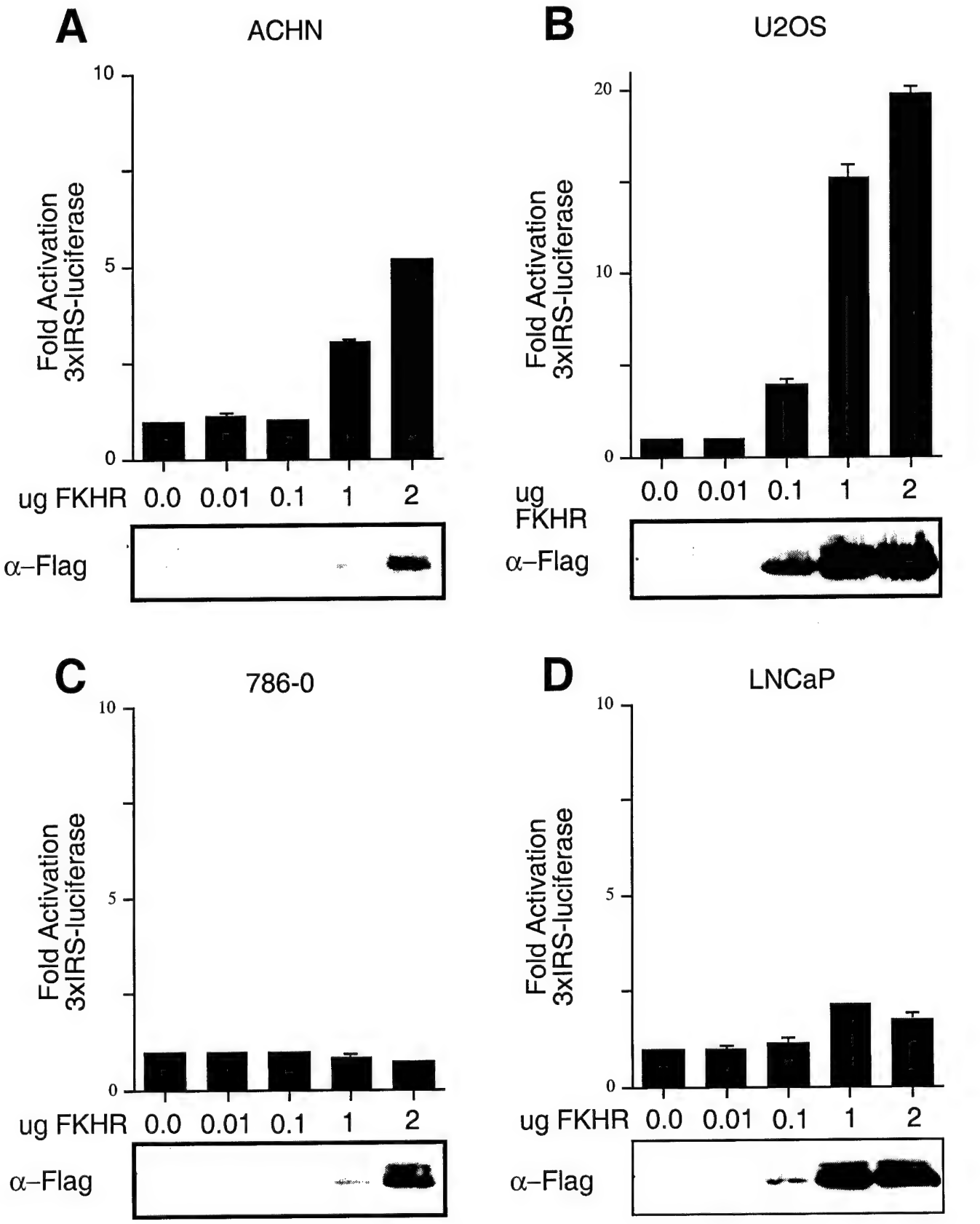

A
ACHN
Fold Activation 3xIRS-luciferase
10
5
0
ug FKHR 0.0 0.01 0.1 1 2
α–Flag
B
U2OS
Fold Activation 3xIRS-luciferase
20
10
0
ug FKHR 0.0 0.01 0.1 1 2
α–Flag
C
786-0
Fold Activation 3xIRS-luciferase
10
5
0
ug FKHR 0.0 0.01 0.1 1 2
α–Flag
D
LNCaP
Fold Activation 3xIRS-luciferase
10
5
0
ug FKHR 0.0 0.01 0.1 1 2
α–Flag

Figure 5

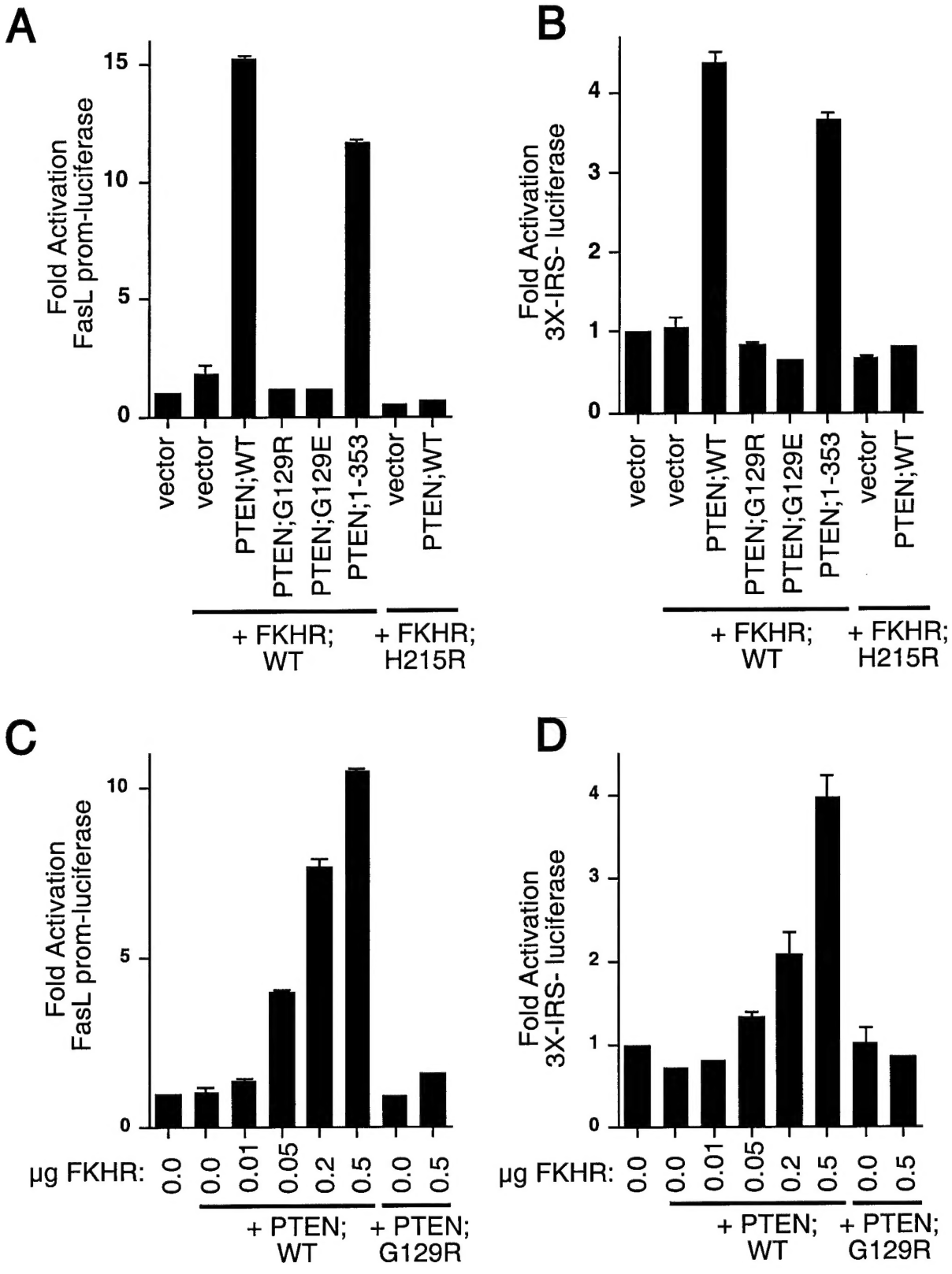

A
Fold Activation
FasL prom-luciferase
15
10
5
0
vector
vector
PTEN;WT
PTEN;G129R
PTEN;G129E
PTEN;1-353
vector
PTEN;WT
+ FKHR; WT
+ FKHR; H215R
B
Fold Activation
3X-IRS- luciferase
4
3
2
1
0
vector
vector
PTEN;WT
PTEN;G129R
PTEN;G129E
PTEN;1-353
vector
PTEN;WT
+ FKHR; WT
+ FKHR; H215R
C
Fold Activation
FasL prom-luciferase
10
5
0
µg FKHR:
0.0
0.0
0.01
0.05
0.2
0.5
0.0
0.5
+ PTEN; WT
+ PTEN; G129R
D
Fold Activation
3X-IRS- luciferase
4
3
2
1
0
µg FKHR:
0.0
0.0
0.01
0.05
0.2
0.5
0.0
0.5
+ PTEN; WT
+ PTEN; G129R

Figure 6

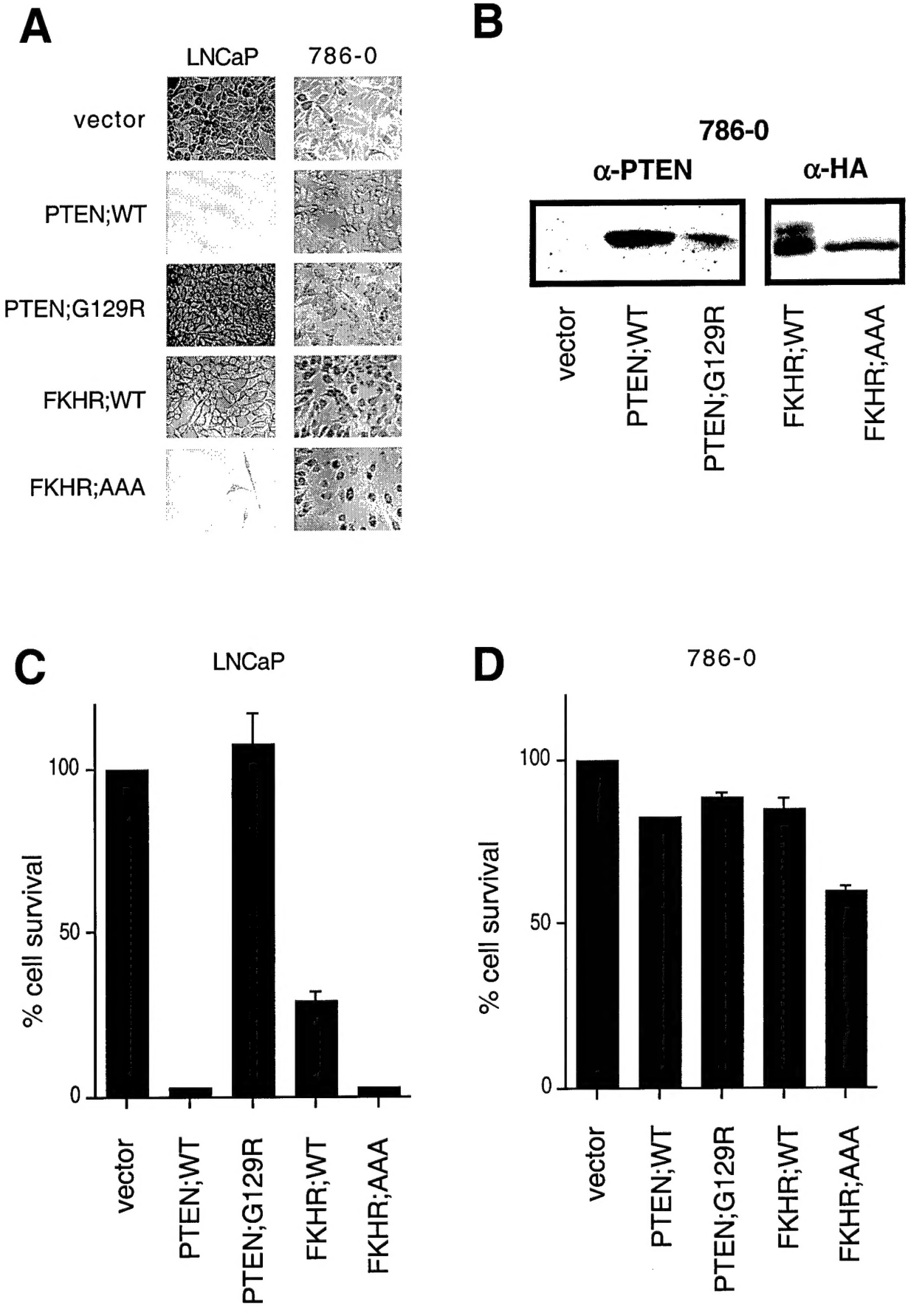

A
LNCaP
786-0
vector
PTEN;WT
PTEN;G129R
FKHR;WT
FKHR;AAA
B
786-0
α-PTEN
α-HA
vector
PTEN;WT
PTEN;G129R
FKHR;WT
FKHR;AAA
C
LNCaP
% cell survival
100
50
0
vector
PTEN;WT
PTEN;G129R
FKHR;WT
FKHR;AAA
D
786-0
% cell survival
100
50
0
vector
PTEN;WT
PTEN;G129R
FKHR;WT
FKHR;AAA

FIGURE 7

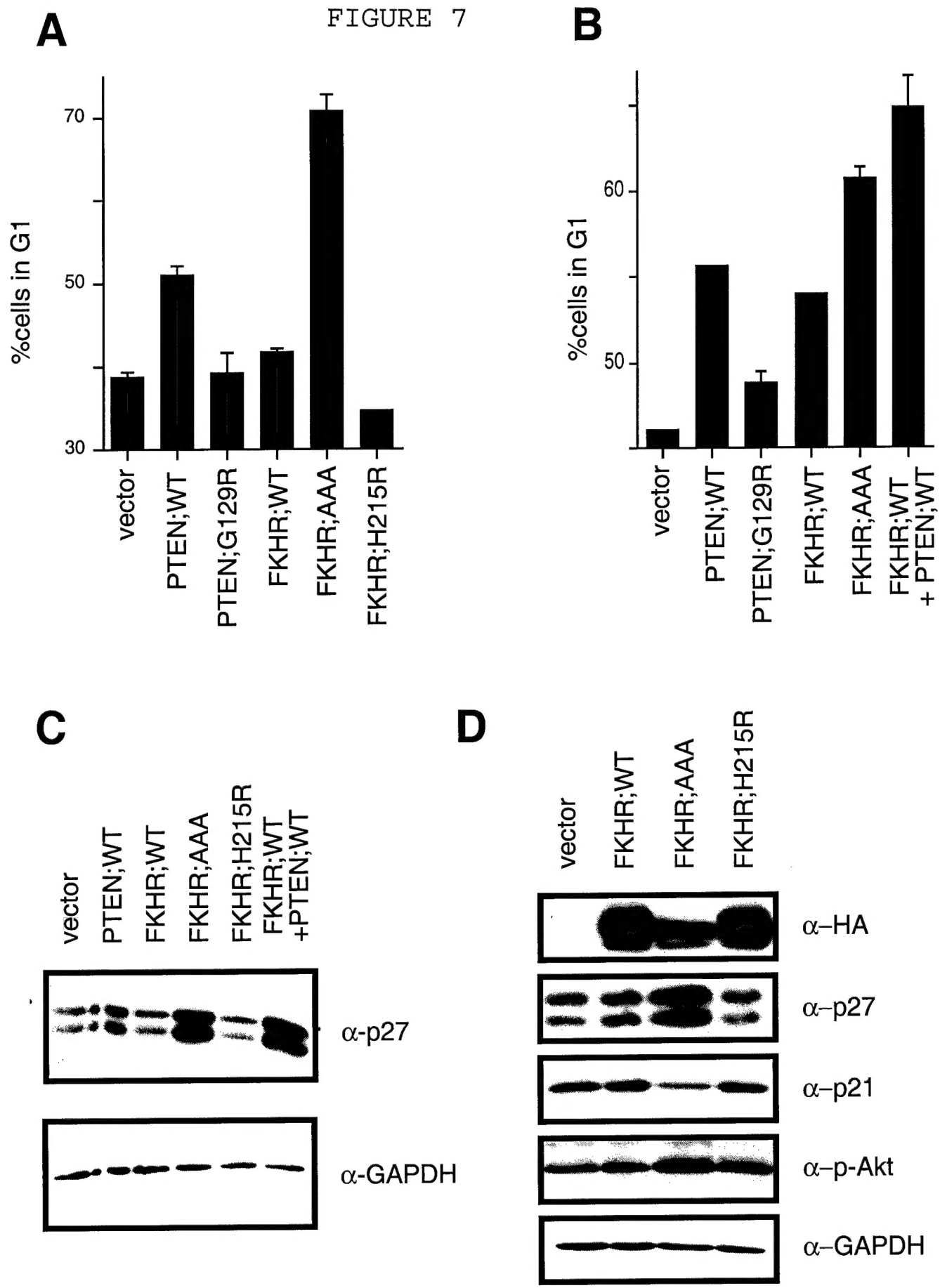
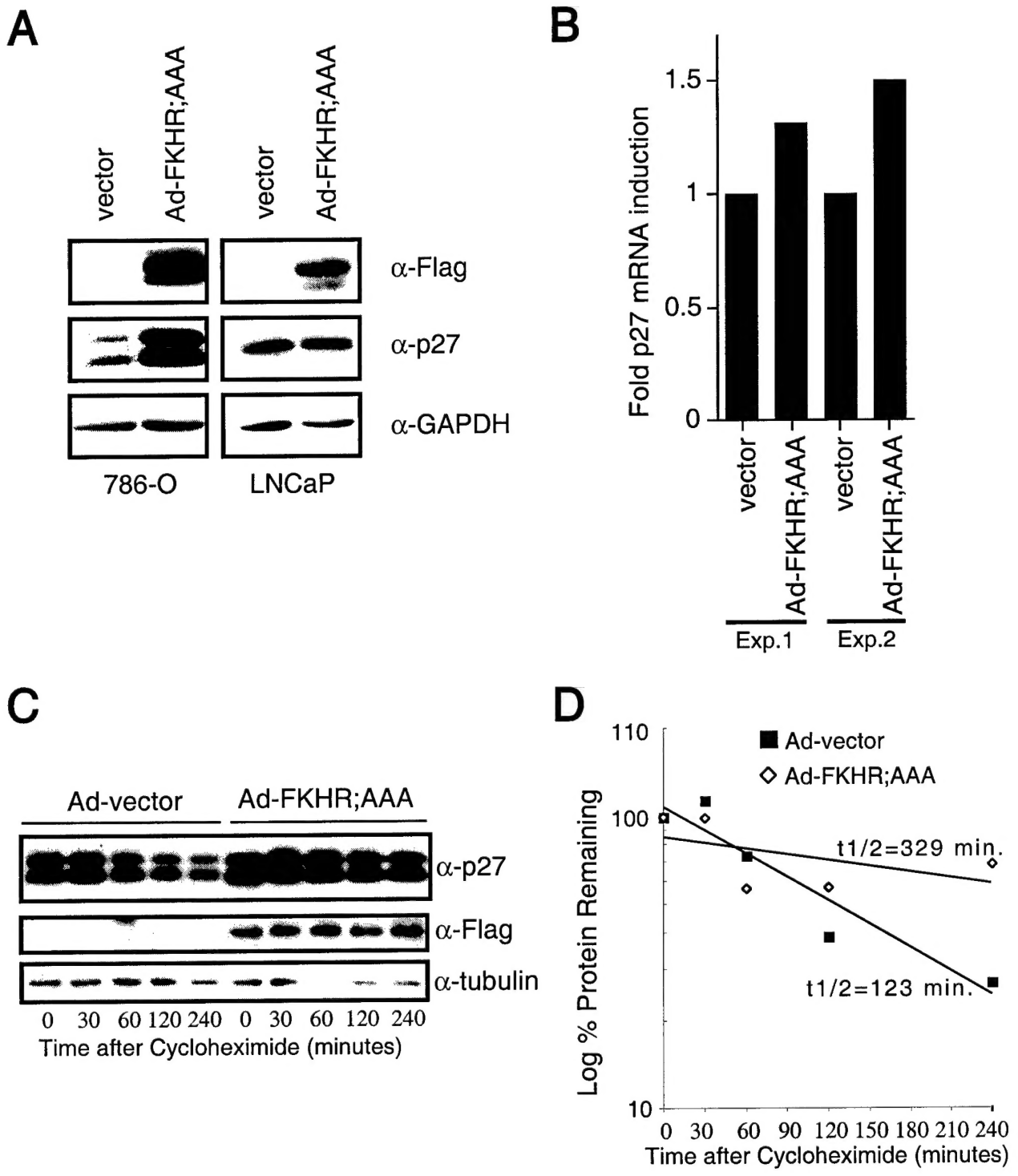

A
%cells in G1
70
50
30
vector
PTEN;WT
PTEN;G129R
FKHR;WT
FKHR;AAA
FKHR;H215R
B
%cells in G1
60
50
vector
PTEN;WT
PTEN;G129R
FKHR;WT
FKHR;AAA
FKHR;WT
+ PTEN;WT
C
vector
PTEN;WT
FKHR;WT
FKHR;AAA
FKHR;H215R
FKHR;WT
+PTEN;WT
α-p27
α-GAPDH
D
vector
FKHR;WT
FKHR;AAA
FKHR;H215R
α–HA
α–p27
α–p21
α–p-Akt
α–GAPDH

Figure 8


A
vector
Ad-FKHR;AAA
vector
Ad-FKHR;AAA
α-Flag
α-p27
α-GAPDH
786-O
LNCaP
B
Fold p27 mRNA induction
1.5
1
0.5
0
vector
Ad-FKHR;AAA
vector
Ad-FKHR;AAA
Exp.1
Exp.2
C
Ad-vector
Ad-FKHR;AAA
α-p27
α-Flag
α-tubulin
0 30 60 120 240 0 30 60 120 240
Time after Cycloheximide (minutes)
D
Ad-vector
Ad-FKHR;AAA
t1/2=329 min.
t1/2=123 min.
Log % Protein Remaining
110
100
10
0 30 60 90 120 150 180 210 240
Time after Cycloheximide (minutes)